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Antimicrobial effect of Essential Oils against pathogenic bacteria and optimization of its formulations combined with other preservative agents

By

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This thesis is dedicated to my mom, Fatemeh Shariat and dad, Eshagh Ghabraie.

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LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
DNA	deoxyribonucleic acid
EO	essential oil
FIC	fraction inhibitory concentration
GRAS	generally recognized as safe
MIC	minimum inhibitory concentration
OAS	organic acid salts
PL	potassium lactate
PPM	part per million
RPM	revolutions per minute
SA	sodium acetate

SUMMARY

Annually around 4 million foodborne illnesses occur in Canada resulting in an economic burden of approximately $3.7 billion. In recent years, several microbiological issues in food safety have emerged. Subsequently, novel techniques and antimicrobial formulations are required to maintain microbiologically safe foods while preserving their natural taste.

Essential oils are one of the best naturally-known candidates to be used as food preservatives due to their inherent antimicrobial properties and Generally Recognised as Safe (GRAS) status. In this study, the antimicrobial activity of 32 EOs was evaluated *in vitro* against *Listeria monocytogenes, Staphylococcus aureus, Escherichia coli, Salmonella* Typhimurium and *Pseudomonas aeruginosa* using 3 different methods (agar diffusion assay, micro-atmosphere assay and broth microdilution assay). Based on the results stemming from the different methods employed in this study, some EOs such as Red thyme, Red bergamot, Winter savory, Chinese cinnamon and Cinnamon bark were found to be more effective than the others as they showed higher antimicrobial activity against the tested pathogenic and spoilage bacteria. In addition, the combination effect of selected EOs was tested based on the checkerboard method. The results showed that the combination of Chinese cinnamon and Cinnamon bark EOs exhibited an additive effect against all the tested bacteria. This combination was selected to perform sensorial analyses which showed that 0.05% was the highest acceptable concentration which did not induce any negative effect on organoleptic properties of ground meat.

The selected combination of EOs at 0.05% was evaluated *in situ* using lean ground pork against the above listed bacterial species. The results showed that this combination of EOs could effectively reduce the bacterial count in a range from 0.47 log (against *L. monocytogenes*) to 0.85

log (against *S. aureus*) after 1 day of storage while the antimicrobial efficiency decreased during the time.

In another related project, multiple barrier technology (hurdle technology) was used to combine several antimicrobial factors at their sub-inhibitory concentrations for food preservation. It was deemed to be a promising way to promote antimicrobial safety without changing the natural taste and smell of food products. The antimicrobial agents were encapsulated in edible polymer to keep the activity of antimicrobial agents during the storage time. In order to find the optimized antimicrobial formulation, the combination of Chinese cinnamon and Cinnamon bark EOs was evaluated with 3 other antimicrobial agents (nitrite, nisin and organic acid salts) *in situ* using fresh pork sausage.

Results showed the combination of 0.025 or 0.05% of EOs with 100 ppm of nitrite, 12.5 ppm of nisin and 1.55 % of organic acid salts reduced *L. monocytogenes* from 1.5 to 3.6 log after 7 days of storage at 4 °C. Sensorial analyses conducted with a panel of 35 trained examiners showed that the selected formulations were organoleptically accepted in both fresh pork sausages and fresh beef sausages in terms of texture, smell and taste.

CHAPTER 1

1. INTRODUCTION

In recent years, due to some changes in life style including consuming ready to cook and ready to eat products, several microbiological issues in food safety have been appeared (Kotzekidou, 2013). Regarding foodborne illnesses several studies reported their results. For instance, Lacroix (2007) reported that foodborne diseases are responsible for approximately 30% mortality of people worldwide. Nesbitt et al. (2014) showed that annually around 4 million foodborne illnesses occurred in Canada which causing an economic burden of approximately $3.7 billion. The Public Health Agency of Canada estimates that each year roughly one in eight Canadians (~ four million people) get sick due to domestically acquired foodborne diseases (Public Health Agency of Canada). Furthermore, in United States around 5 to 86 billion dollar is spent in the treatment and prevention of foodborne illnesses (Lacroix, 2007). In the European Union, the foodborne infections mainly caused by bacteria such as *Listeria* and *Salmonella* result in greater than 380,000 infections annually(García et al., 2010). Food can be contaminated during storage, handling (preparation), and display or even after cooking (post contamination) due to improper handling.

Despite the recent advances in technologies for controlling foodborne pathogens, the number of foodborne illnesses has increased in recent years which demonstrate a need for new techniques or new antimicrobial formulations to eliminate pathogenic bacteria. Moreover, due to an increase in consumption of more ready to cook or ready to eat products such as packed fruits and vegetables, we have witnessed an increase in the number of foodborne disease outbreaks which makes it necessary to find other ways to control bacterial contamination. Indeed, there is an increased demand by consumers for high quality, microbiologically safe and natural tasting

foods. This has lead food companies toward using natural antimicrobial agents at low level concentrations in order to prevent bacterial growth without affecting the organoleptic qualities of food. Hence, nowadays food preservation and food safety are the key concerns of food companies.

2. LITERATURE REVIEW

2.1. Microbiology of meat

Due to possessing all necessary amino acids and bio available minerals, meat and meat products have an important place in consumers' diets. Ground meat is a complex food system which possesses soluble carbohydrates, proteins, endogenous enzymes and other factors that support the growth of bacteria. Ground meat has a short shelf-life as the mentioned factors make the meat highly perishable, so preservation technology is necessary (Dave and Ghaly, 2011; Mello da Silveira et al., 2014; Zhou et al., 2010).

Meat is among the most susceptible foods to microbial contamination. *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* are among the most dangerous microorganisms which could be associated with meat (Hernández-Ochoa et al., 2011). Several foodborne diseases are caused by consuming undercooked meat. For instance, the most common major risk factor involving *L. monocytogenes* is the consumption of undercooked ground beef (Solomakos et al., 2008b). Nevertheless, the consumption of properly cooked fresh sausage is considered safe (Mello da Silveira et al., 2014). Since meat contamination can cause illnesses and food spoilages, antimicrobial agents are needed in processed meats to control the natural spoilage process by inhibiting the growth of undesirable microorganisms or controlling their development (Tajkarimi et al., 2010).

In this study, fresh pork sausage was used mainly as a food model. The term "fresh meat" is used for recently processed meat without any treatment except chilling (Zhou et al., 2010).

2.2. Food pathogens which they used in this study

In this study, *in vitro* and *in situ* antimicrobial activity susceptibility was determined using five foodborne pathogens and spoilage bacteria. *Listeria monocytogenes, Staphylococcus aureus, Escherichia coli, Salmonella* Typhimurium and *Pseudomonas aeruginosa* were chosen to be used in this study. Consumption of contaminated food with these bacteria can causes foodborne illness which is one of the big concerns of public health. Oussalah et al. (2007) showed that foodborne pathogens such as *Salmonella* sp., *Staphylococcus aureus, Listeria monocytogenes* and *Escherichia coli* caused the numerous illnesses and death. According to Arslan et al. (2011) and Gutierrez et al. (2009), *Pseudomonas aeruginosa* can cause food spoilage which is one of the reasons for off-flavour and discoloration of refrigerated meat.

2.2.1. Listeria monocytogenes

Listeria is Gram-positive bacteria. It is a facultative intracellular foodborne pathogen which causes listeriosis. This bacterium is one of the biggest concerns of public health as it can be found everywhere in nature like in domestic animals, birds, insects, meat, fish, dairy products, vegetables and soil. It can even be found in 5% of healthy people's intestines. Thus, food can be easily contaminated with this bacteria (Ramaswamy et al., 2007). *Listeria* can be also detected in cooked food and pasteurized milk due to post contamination or not achieving adequate temperature for cooking. (Kotzekidou, 2013; Ramaswamy et al., 2007). Products having a shelf-life longer than 5 days are highly susceptible to contamination and cause listeriosis as the presence of *L. monocytogenes* in these foods can reach to levels detrimental to human health.

Due to the serious diseases which *Listeria* causes, this bacterium is known as one of the biggest concerns of public health. The growth capability of *Listeria* makes this bacterium hard to control. They are able to grow both aerobically or anaerobically and also while most bacteria cannot grow below 4°C, *Listeria* grows in a wide range of temperatures (−4°C and 50°C) and it could survive and grow easily at refrigerated temperature (Cammack et al., 1999; Kotzekidou, 2013; Ramaswamy et al., 2007).

Consuming contaminated food with *L. monocytogenes* is dangerous especially for susceptible individuals such as pregnant women and fetus, elderly people, and people with weakened immune system such as cancer and organ transplant patients (Ramaswamy et al., 2007). *L. monocytogenes* can survive in the acidic pH of the stomach and can go through the small intestine to the liver. There, they will be multiply and affect the central nervous system and cause severe diseases such as meningitis (Ramaswamy et al., 2007). In fact, 20-30% of infections have high risk and may become fatal (Ramaswamy et al., 2007). In the USA there are 1600 case of listeriosis with 400-500 deaths annually (Ramaswamy et al., 2007).

2.2.2. Staphylococcus aureus

Staphylococcus aureus is a Gram-positive coccal bacterium which is frequently found in the human respiratory tract and on the skin of about 25% of healthy people and animals. It can grow in a wide range of temperatures (6 to 48°C) but grows best at 37°C. *S. aureus* is a common cause of skin infections, respiratory disease, and food poisoning. *S. aureus* can survive from hours to weeks, or even months, on dry environmental surfaces, such as cooked meat, dry-fermented sausage, ham and generally in foods with reduced water activity. Most of the food contaminations can occur due to mishandling and cross-contamination during preparation. The presence of *S. aureus* does not always indicate infection as it is an opportunistic pathogen

(Kotzekidou, 2013). Consuming contaminated food with Staphylococcal toxins causes staphylococcal food poisoning which is a gastrointestinal illness. In the US alone, around 1,200 deaths due to staphylococcal food poisoning are reported annually (Mead et al., 1999). Meat, puddings, and sandwiches are at highest risk of contamination and can cause staphylococcal food poisoning as they are prepared by manual manipulation. *S. aureus* could be resistant to antibiotics such as penicillin and methicillin. Some EOs such as tea tree, *Origanumvulgare*, *Mentha piperita*, and, *Hofmeisteria schaffneri* oil are effective against *S. aureus.* It has been demonstrated that Tea tree oil has an activity against methicillin-resistant *S. aureus* (Oussalah et al., 2006; Solorzano-Santos and Miranda-Novales, 2012). Indeed, Solorzano-Santos and Miranda-Novales, (2012) demonstrated that the combination of EOs with other antimicrobial agents was highly effective against multi-drug-resistant *S. aureus* (Solorzano-Santos and Miranda-Novales, 2012)

2.2.3. Escherichia coli

Escherichia coli is a Gram-negative, facultatively anaerobic bacterium which commonly found in the lower intestine of warm-blooded organisms. If oxygen is absent, it is able to switch to fermentation or anaerobic respiration. Most *E. coli* strains are harmless and are part of the normal flora of the gut, which produce vitamin K_2 but the others can cause serious food poisoning. *Escherichia coli* O157:H7 is an important pathogen which causes a severe foodborne illness. The bacteria can be transferred to the outer surface of meat during butchering. Processing can then spread the bacteria throughout the meat. Consuming undercooked ground beef contaminated with *E. coli*, usually followed by upset stomach from which the affected individual usually recovers, but sometimes the infections could be life threatening such as severe anemia or kidney failure, which can lead to death. Apart from meat, raw milk or dairy products, fruits and vegetables are also susceptible to get contaminated with *E. coli*.

E. coli could easily become resistant to antibiotics so it is important to inhibit this bacterium. Geraniol which can be found in several EOs such as lemon, wild bergamot and geranium reveal high activity in modulating drug resistance of *E. coli* (Solorzano-Santos and Miranda-Novales, 2012). Around 73,000 cases of infection per year estimated in USA. A study published in 2005 estimated the annual cost of *E. coli* O157:H7 illnesses to be $405 million (http://www.about-ecoli.com)

2.2.4. Salmonella Typhimurium

Salmonella Typhimurium is a pathogenic Gram-negative bacterium which commonly found in the intestines of animals and birds. Its toxicity is due to an outer membrane consisting largely of lipopolysaccharides (LPS). The bacteria can be transmitted to people when they eat foods contaminated with animal feces (zoonotic) (Bajpai et al., 2012). The infection is usually caused by eating raw or undercooked meat, poultry, eggs or egg products. Food might get contaminated during food processing or food handling by the unwashed hands of an infected food handler. Beef, poultry, milk, and eggs are most often infected with *Salmonella* but they usually look and smell normal. It is reported that doses as low as 10 microorganisms or even fewer can cause illness (Kotzekidou, 2013).

Food contaminated with *Salmonella* can cause salmonellosis. *Salmonella* will cause mild to serve infections (Bajpai et al., 2012). Most salmonella infections can be classified as gastroenteritis. Salmonellosis is among the third major cause of foodborne acute gastroenteritis (Mello da Silveira et al., 2014). *S.* Typhimurium causes gastroenteritis in humans and other mammals (Nazer et al., 2005). The diarrheal illnesses is mostly recorded in industrialized countries (Kotzekidou, 2013). Young children, older adults, and people with weakened immune systems are the most likely to experience severe infections (Bajpai et al., 2012). In a small number of

cases, *Salmonella* might spread from the intestines to the blood and other part of body. In this way it causes severe illness and in vulnerable people, death.

Salmonella is resistant to antibiotics. The overuse of antibiotics in the food industry contributes to the spread and the emergences of bacterial resistant to antibiotics. (Bajpai et al., 2012). It is estimated that salmonellosis affects around 40,000 in the United States annually (Jung et al., 2009).

2.2.5. Pseudomonas aeruginosa

P. aeruginosa is a Gram-negative, coccobacillus, aerobic and facultative anaerobe bacterium which can be found in soil, water, plants, animals, and can be transmitted to human through food and water (Neves et al., 2014).

P. aeruginosa is a human opportunistic pathogen which generally infects the pulmonary tract, urinary tract, and causes blood infections (Arslan et al., 2011). This bacterium can rapidly become resistant to antibiotics by mutation or horizontal gene transfer of antibiotic resistance determinants (Solorzano-Santos and Miranda-Novales, 2012).

Pseudomonas spp. is one of the major food spoilage bacteria due to their extracellular enzymes which include proteases, lipases, and lecithinase. As an example, this extracellular enzyme of *P. aeruginosa* could spoil the milk by degrading carbohydrates, proteins, and fats of milk and give bitter flavour to cheese. The quality of food should be for human consumption (Arslan et al., 2011).

The bacteria can be spread within hospitals by workers, medical equipment and food. During food production when chilling treatment is not applied properly, the problem of food spoilage becomes more serious (Arslan et al., 2011). *P. aeruginosa* whether spoilage or pathogenic can

grow in food because of its high nutritional value, water content and neutral pH found in some food such as dairy products (Arslan et al., 2011). *P. aeruginosa* can cause food spoilage and become pathogenic for humans as a second infection and is a serious opportunistic human pathogen as it is resistant to several antimicrobials (Arslan et al., 2011).

Annually, in United States, around 51,000 *P. aeruginosa* infections occur of which approximately 13% are multidrug-resistant and cause roughly 400 deaths.

(http://www.cdc.gov/hai/organisms/pseudomonas.html)

2.3. Antimicrobial agents used in this study

In this study, Essential Oils (EOs), nisin, nitrite and organic acid salts were used as antimicrobial agents to control the growth of the target bacteria. These compounds were used in sausage preparation. Our study showed that each of these compounds alone have antimicrobial activity, so by combining all together at their sub-inhibitory concentrations, it would be possible to inhibit microbial growth without altering the natural taste and smell of food products.

2.3.1. Nitrite

Nitrite is an antimicrobial agent used in food which extends the shelf life of meat. It also contributes to color stability along with improving sensory quality of meat products by giving unique color, texture and flavor (Cui et al., 2010; Sindelar and Milkowski, 2011). It has been found that nitrite improves sensory qualities of meat through formation of NO-myoglobin that gives a red color to the meat (Honikel, 2008).

Sodium nitrite or potassium nitrate has been used as the main source of nitrite (NO_2-) since the 19th century to preserve the food especially meat and meat products; however, sodium nitrate

($NaNO_3$) is typically used in fermented sausages which require longer preservation times (Nyachuba et al., 2007).

In fact the compounds derived from nitrite during storage time are bactericidal compounds not nitrite itself (Cammack et al., 1999). Adding nitrite to food causes the formation of nitrite oxide which causes inhibition of the phosphoroclastic system, then as a consequence, intracellular ATP will decrease rapidly resulting in death of the cells (Cui et al., 2010).

Nitrite is used in meat products to prevent the growth of heat resistant spores of *C. botulinum* but due to health concerns, is used at the lowest effective concentration (Sindelar and Milkowski, 2011). Moreover, it has been found that dietary nitrate (i.e. from vegetables and fruits) is a source for producing nitrite and nitric oxide in the body which affects normal body functions. Furthermore, nitric oxide plays a role in controlling blood pressure, immune response, wound repair, and neurological functions (Hunault et al., 2009; Sindelar and Milkowski, 2011) .

Despite its widespread usage it has some disadvantages such as being a very reactive substance which initiate several chemical reactions (Davidson et al., 2010). Nitrite could be carcinogenic as it has been shown to induce mutations in some bacteria like *S.* Typhimurium. Due to the acidic environment of the stomach, production of carcinogenic nitrosamines could occur (Davidson et al., 2010; Honikel, 2008). Hence the use of nitrite is strictly regulated. The highest concentration of nitrite salt in food should be less than 200 ppm (Cui et al., 2010). In meat products, nitrate can be reduced to nitrite by the bacteria which are present in meat or by adding bacteria which produce the nitrite reductase enzyme. Furthermore, nitrate can be reduced to nitrite in the oral cavity, so the sum of both nitrite and nitrate should be controlled for human consumption

(Honikel, 2008). Balancing the risks versus the benefits for food preservatives is always essential (Davidson et al., 2010).

2.3.2. Nisin

Numerous bacteria produce substances of proteinaceous structure with antimicrobial activity. Nisin is a ribosomally synthesised antibacterial polypeptide with 34 amino acid residues which has been used as a food preservative. Nisin is a heat-stable cationic peptide produced by Lactic Acid Bacteria (LAB). Compared to other bacteriocins such as pediocin, nisin is the only bacteriocin that has been approved as food additive; however, both have an antimicrobial effect. Nisin attracted attention and became the most thoroughly studied bacteriocin due to the fact that it is a safe additive for food and has the GRAS status. Moreover, nisin can be degraded by proteolytic enzymes which can be found in mammalian gastrointestinal tract, so it is safe for human use (Zacharof and Lovitt, 2012).

Jones et al. (2005) reported that the FAO/WHO recognized nisin as a food preservative in 1969 and it is the only bacteriocin which has been used in food industry and currently it is widely used in more than 50 countries. Generally, bacteriocins can work against closely related species but nisin is a "broad-spectrum" bacteriocin which is effective against many Gram-positive organisms and also effective against spores. It has sporostatic effect and can delay the spore outgrowth (Wijnker et al., 2011) Through binding to anionic lipids which exist in membranes of Gram positive bacteria, nisin causes the formation of pores in the membrane (Zacharof and Lovitt, 2012). Indeed, FDA approved the usage of nisin against *C. botulinum* in canned products (Abdollahzadeh et al., 2014; García et al., 2010; Jones et al., 2005; Millette et al., 2007; Solomakos et al., 2008a). Besides, it is active against pathogenic and food spoilage bacteria as well as *S. aureus* and *L. monocytogenes* (Zacharof and Lovitt, 2012). In the food industry, nisin

is obtained by fermentation from the culturing of *Lactococcus lactis* on natural substrates, such as milk and it is not chemically synthesized.

2.3.3. Organic acid salts

Organic acids and their salts are used as preservatives in foods to increase the lag phase of microbial proliferation. Sodium salts of the low molecular weight organic acids, such as lactic acid, slow the growth of spoilage bacteria and increase the shelf-life while marinating, promoting the organoleptic quality of sausages (Crist et al., 2014; Ibrahim Sallam, 2007).

Potassium lactates and sodium acetate are two of the GRAS substances which are considered as antimicrobial agents and are widely used as preservatives to prolong the shelf-life and also increase the safety of meat products. Potassium lactate is a liquid derived from lactic acid that is naturally present in animal tissue. It extends the lag phase of pathogenic bacteria resulting in the extension of food shelf-life. In fact lactates can inhibit the growth of bacteria by reducing the water activity of food products followed by retarding the development of bacteria and also by acidifying the intracellular pH (Stekelenburg, 2003).

Ibrahim Sallam (2007), showed the high antimicrobial activity of sodium acetate compared to sodium lactate and sodium citrate. These organic salts have a suppressing effect on the growth of various pathogenic and spoilage bacteria and these are economical as well (Ibrahim Sallam, 2007). In fact, sodium acetate has been shown to delay lipid oxidation thereby prolonging the shelf-life of food during refrigerated storage (Ibrahim Sallam, 2007). Sodium acetate is approved by the USFDA as a flavouring and pH control agent. Several studies have demonstrated the antimicrobial activity of sodium acetate in different food systems. Manju et al. (2007) used 2% of sodium acetate and combined it with vacuum-packaging and found an extension of the shelf-life of seafood by 15 days. In addition Ibrahim Sallam (2007) showed that an emulsion

containing 2.5% of sodium acetate was able to prolong the shelf-life of sliced salmon up to 15 days.

Sodium diacetate which is the mixture of Sodium acetate and acetic acid have also been used in food to control the pH, improve the sensorial quality and promote food safety (Stekelenburg, 2003).

2.3.4. Essential Oils (EOs)

During evaluation, plants have produced EOs to defend themselves against predators (fungi, insects, etc.) and microbial pathogens (Bassolé and Juliani, 2012). Essential Oils or herbal extracts are mostly extracted from the plants from warm climates like those found in tropical or Mediterranean countries (Bakkali et al., 2008). EOs are mainly produced from aromatic plants through extraction from nonwoody organs. EOs are mostly liquid at room temperature and their color is ranging from pale yellow to emerald green and from blue to dark brownish red (Bassolé and Juliani, 2012; Dorman and Deans, 2000). They have an oily consistency and can be made by different tissues of a single plant such as stems, leaves, flower, buds, seeds, fruits, and roots (Bakkali et al., 2008). They should be preserved in dark and airtight containers to prevent the changes of EOs components and evaporation (Burt, 2004).

EOs have been used since ancient periods and have been used for various reasons (Bakkali et al., 2008; Porres-Martínez et al., 2013; Solorzano-Santos and Miranda-Novales, 2012). The very first usage of EOs dates back to 16^{th} century where people use myrrh with honey to inhibit the bacterial growth. Due to their compositions and the concentration of each of their components the properties and activities of EOs are different. They have antiseptic properties such as antibacterial, antifungal, insecticidal activities (Bassolé and Juliani, 2012). These properties are necessary for EOs as they are prepared to defend against predators and herbivores. Most of the

EOs have cytotoxic affects without being mutagenic or carcinogenic (Bakkali et al., 2008). Antimutagenic properties could be due to various affects such as inhibiting the entry of the mutagen, inactivation of the mutagen, and activation of the cell to produce antioxidants (Bakkali et al., 2008). Over 1340 plants have been identified with antimicrobial compounds (Tajkarimi et al., 2010).

Around 3000 EOs are known and 300 of them are commercially used in perfumes, dentistry, agriculture, and food products. They have a strong aroma and are also used in cosmetic applications (Bakkali et al., 2008). EOs are used in creams and lotions for treatment of some skin diseases or for cosmetic use (Solorzano-Santos and Miranda-Novales, 2012). Cinnamon, Clove, Mustard, Garlic, Ginger and Mint are traditionally used in health remedies in Asian countries (Tajkarimi et al., 2010). Indeed there are numerous studies which were conducted to show the antimicrobial activity of plant origin compounds. EOs are one of the best antimicrobial candidates for using as preservatives in food system (Tajkarimi et al., 2010).

2.3.4.1. The factors that can change the EOs properties

Plant density, age, climate, region, soil composition, harvesting season, the parts of the plant used to extract the EOs, and also methods used in their distillation are the factors that can affect the properties of EOs (McGimpsey et al., 1994; Oussalah et al., 2007; Lacroix, 2007). The antimicrobial activity of each EO should be checked and it is not possible to assume all the EOs from same type of plant have the same level of activity. Screening the EOs to select the most active one is highly important (Dussault et al., 2014).

2.3.4.2. Composition (major and minor compounds)

EOs are very complex mixtures. They can contain around 20-60 different components. To chemotype the EOs, chromatography and mass spectrometry are used (Bakkali et al., 2008). The

components of EOs are derived from these chemical groups: terpenes, terpenoids, and aromatic compounds (Laird and Phillips, 2012). In fact Terpenoids are the terpenes oxygen and can be subdivided into alcohols, esters and phenols (Jayasena and Jo, 2013; Solorzano-Santos and Miranda-Novales, 2012).

Both major and minor compounds can contribute to the antimicrobial properties of EOs. The main effect of EOs is attributed to their major compound; however, the minor compounds could also have synergetic or additive activity with the major ones (Bassolé and Juliani, 2012; Burt, 2004; Hyldgaard et al., 2012; Oussalah et al., 2007; Turgis et al., 2009). As the antimicrobial activity of EOs is attributed to different mechanisms so using the whole EOs could demonstrate more antimicrobial activity than their major or minor components alone. Therefore it is better to examine the EOs as a complex mixture instead of just surveying the antimicrobial effect of their main compounds such as carvacrol, and thymol (Bassolé and Juliani, 2012; Turgis et al., 2009).

2.3.4.3. Mechanism of action of EOs

The lipophilic character of the components of EOs contribute to their antimicrobial effect as they are able to be accumulated in lipidic bilayer of cell membrane, which will follow the loss of ions and decreasing the ATP and cause cell death (Bakkali et al., 2008; Oussalah et al., 2006; Quirós-Sauceda et al., 2014). The hydrophobicity of EOs enables them to go through the cell membrane and mitochondria to make them more permeable. Then, their high permeability makes the cells more sensitive to other antimicrobial agents or due to extensive leakage of critical molecules, the cell will die (Solorzano-Santos and Miranda-Novales, 2012). In another study, scanning electron microscopy showed a significant decrease in unsaturated fatty acids while the quantity of saturated fatty acids increased due to the usage of EOs (Bakkali et al., 2008). Moreover, EOs contain hydroxyl group and it may prevent the genetic material synthesis (Hernández-Ochoa et

al., 2011). Indeed some component present in EOs could bind to proteins and inhibit the activity of metabolic enzymes and cause cell death (Lacroix, 2007). Making the membrane more rigid, depolarizing the membrane, reducing respiratory activity, and coagulating of cytoplasmic material are some other ways that EOs can inhibit the bacteria (Hyldgaard et al., 2012).

2.3.4.4. EOs in food

Although EOs have GRAS status and have shown the promising antimicrobial effects, their application is limited due to their strong taste and odor. Compared to *in vitro* system, they should be used at higher concentration in food system to cause the same inhibition activity but at high concentration EOs will change the organoleptic properties of food.

2.3.4.5. Interaction of EOs with food matrix

Food ingredients can influence the efficiency of EOs. Moreover, in low water foods the antimicrobial efficiency of EOs might be reduced. Some studies demonstrated the negative effect of high quantities of fat and protein on EOs efficiency (Celikel and Kavas, 2008). However, Gutierrez et al. (2008), showed the presence of proteins in food could promote the activity of EOs.

2.3.4.6. EOs in combined treatments

The combination of EOs with another compound could change the antimicrobial activity of EOs. These compounds could be a bacteriocin like nisin, another EO, or even some other compound such as nitrite, etc. The effect of these interactions on the antimicrobial activity of EOs could be synergistic, additional or antagonistic. For instance, Lacroix (2007) reported that sodium chloride, sugars and organic acids might have synergistic effect with EOs.

2.4. Technologies for preservation

Preservation technologies have been used for long time. Cooling down, smoking, salting, drying and etc are common technologies which use to prolong the shelf life of meat (Zhou et al., 2010). In general, meat preservation methods could be classified in three main groups temperature, moisture reduction and direct targeting the microorganisms (Zhou et al., 2010).

2.4.1. Control by temperature

Temperature can control the growth of bacteria or eliminate them if it is below or above the optimum range for bacterial growth. In case of fresh meat, refrigeration has been traditionally used as a preservation method. Indeed hot smoking seals the outer layer of food and cooks the surface of meat a little so it would be more difficult for bacteria to penetrate it. Cold smoking means the food should be dried quickly (Zhou et al., 2010).

2.4.2. Control by moisture

Usage of salt as a preservative dates back to as early as 3,000 B.C. It decreases the water activity and develops osmotic pressure which draws water out of the microorganism, and slows the rate of oxidation. However, the concentration of NaCl should be at least around 20% (Sindelar and Milkowski, 2011).

2.4.3. Direct effect on microorganism

Another technique to preserve the food is to attack the bacteria with antimicrobial agents to inhibit their growth and extend the shelf life of food. Antimicrobial agents can kill bacterial cells or delay in their growth *via* various ways such as membrane permeabilization and inactivation of enzymes (Lacroix, 2007).

2.4.3.1. Irradiation

Since 1940, ionising radiation has been recognized as a method of direct microbial inhibition for preserving meat (Zhou et al., 2010). Gamma irradiation was used in this study used mainly to

sterilize the meat samples. Ionizing radiation is an easy and reliable technology for improving the microbial safety and shelf life of food (Sales et al., 2012). As irradiation can dislodge electrons from atoms and create ions, it has been named ionizing radiation. Radiation can kill microorganisms and viruses by damaging DNA and produces peroxides which is a powerful oxidizing agent in cells.

Figure 1. The international Radura logo which is used to show a food has been treated with ionizing radiation.

Ionizing radiation can control the microorganisms without raising the temperature significantly so it also called cold pasteurization (Alighourchi et al., 2014). It controls and inactivates spoilage and pathogenic bacteria, mold and yeast and prolongs the shelf life of fresh fruits and vegetables

Studies have demonstrated that ionizing radiation can effectively inactivate pathogenic microorganisms in water, food and medical products (Jebri et al., 2013).

The World Health Organization (WHO), the Center for Disease Control and Prevention (CDC), the United State Department of Agriculture (USDA) and FDA have approved the safety of irradiation so it has been used in around 56 countries (Alighourchi et al., 2014).

Already some foods such as onions, potatoes, and ground spices have been irradiated and are allowed to be sold in Canada. However, foods containing more than 10 % irradiated ingredient should display the international radiation symbol (Fig.1).

Gamma radiation inactivates the bacteria by damaging the DNA either directly by breaking the nucleic acid or indirectly by radiolysis of water and preparing hydroxyl radicals (Jebri et al., 2013; Sommer et al., 2001).

In this study Gamma irradiation was used for sterilizing the samples. Gamma rays have a shorter wavelength than ultraviolet light. Before each *in situ* experiment, all of the sausages were irradiated at the Canadian Irradiation Center at 45 kGy using a UC-15A irradiator (MDS Nordion International Inc., Kanata, Ontario, Canada) equipped with a ^{60}Cobalt source.

(http://www.inspection.gc.ca/food/information-for-consumers/fact-sheets/irradiation/eng/1332358607968/1332358680017)

2.5. Using the best technology (Hurdle technology)

To assure optimum safety without affecting organoleptic properties of food, novel antimicrobial control methods (Hurdle technology) should be established (Cui, Li, et al., 2011). Hurdle technology uses the combination of mild food processing factors together to get acceptable safety and high sensory qualities (Cui et al., 2010).

Consumers demand for natural and high sensory quality of food means limited usage of preservatives and low thermal processing. Generally the preservation method should be energy saving, environmental friendly, organoleptically acceptable and especially highly effective to inhibit the pathogens (Zhou et al., 2010). To preserve the fresh meat it is best not to use thermal treatment and instead get benefits from other technologies such as Hurdle technology (Zhou et al., 2010). Mild preservation technologies are important for modern food industries and by combining these processes organoleptic quality will improve. Hurdle technology or the combination of different processes can be used to achieve microbial safety as it is probable that

using one antimicrobial agent eliminates one organism but provide a good condition for other microorganisms.

2.6. Encapsulation in edible polymer

To extend the shelf life, it is necessary to enhance and stabilize the microbiological safety which means the growth of bacteria should be controlled. Using either natural or synthetic antimicrobial agents has some limitations. They would impart off flavors, or could be degraded by food ingredients and lose their activity in short time. Indeed most of the food additives are temperature sensitive (Quirós-Sauceda et al., 2014).

The term edible coating is generally used for a thin edible layer applied to the surface of foods, but it also could be used as a matrix to entrap bioactive compounds such as antimicrobials. Encapsulating the antimicrobial factor in edible polymer provides some benefits when compared to antimicrobial dips and sprays. Dipping and spraying methods are not suitable for long term storage as diffusion of the factor would continue into the food and may allow microbial growth on the surface while encapsulation will delay the migration of the agent. So with this promising technique, the risk of pathogens growing on the surface of food will be reduced and the food shelf life will be extended. In addition, the change in the organoleptic properties of the food after this treatment is minimal (Quirós-Sauceda et al., 2014).

The structural material of edible polymer could be composed of proteins (gelatin, bulk proteins, zein), polysaccharides (starch, alginate, chitosan) and lipids (glycerol, waxes, and esters) alone or in combination. The barrier properties of these polymers depend on the types of compound used in polymer and also the strength of different kinds of binding (covalent bonds, H-bonding and ionic bonds) between coating-forming polymer molecules. Physical and chemical treatment can cause changes on these properties. For instance, chemical treatments, including the use of

emulsifiers can modify interfacial energy at the interface of immiscible system (ie. Water-lipid interface). Besides, physical treatments, such as irradiation and heating, can also promote the cohesive strength of the coating through the formation of cross-links (Quirós-Sauceda et al., 2014).

Polysaccharides such as alginate are generally hydrophilic and this is mostly due to the presence of large number of hydroxyl groups which shows the importance of H-bonds in film formation (Quirós-Sauceda et al., 2014). To prepare a polysaccharide-based polymer, during the co-acervation process, the long chains of the polymer must be disrupted and new hydrogen bonds must form to create the matrix (Quirós-Sauceda et al., 2014). Encapsulation of antimicrobial agents in edible polymers brings us notable benefits such as controlled release, preservation of the natural taste, promotion of solubility, and preservation of bioactivity during the process and during storage time.

2.6.1. Controlled release

Entrapping the antimicrobial compound in a polymeric matrix retards their release. As a result the antimicrobial agent will last for longer times and following of that, the shelf life of food would be extended (Neetoo et al., 2010; Quirós-Sauceda et al., 2014). Edible coatings control the release of encapsulated compound via different ways such as, melting, diffusion, degradation, or particle fracture (Quirós-Sauceda et al., 2014).

The melting of the polymer could cause the slow release of antimicrobials. In some cases the encapsulated compounds are soluble in coating and that controls their release. Either diffusion or degradation or the effect of both causes the release of the encapsulated compound from a matrix-type delivery system. Relative humidity (RH) also has a positive effect on the release of volatile compounds. In some cases, the adsorption of water in high HR destroys the capsule. It has been

shown that high temperature can increases the rate of release of some compounds (Quirós-Sauceda et al., 2014).

2.6.2. Keeping the natural taste

The release of antimicrobial compounds can be is controlled by encapsulation, which reduces the probable negative organoleptic effect of each compound (Neetoo et al., 2010; Quirós-Sauceda et al., 2014).

2.6.3. Promotion of solubility

Most antimicrobial agents inhibit bacteria by damaging their cell membrane, inactivate cellular enzymes, or act in some other ways but the element which is essential for all of them is to contact the bacterial cell directly. Encapsulation improves the solubility of antimicrobial compounds and makes them available in the whole food matrix (Neetoo et al., 2010; Quirós-Sauceda et al., 2014). Encapsulation effectively increases the solubility of additives in the food matrix. For instance, liposomes are consisting of lipid bilayers, so they can encapsulate or bind a variety of molecules. In this way they increase the fat solubility of compounds in food matrix(Quirós-Sauceda et al., 2014).

2.6.4. Preservation of bioactivity

The food component can reduce the antimicrobial activity of added and naturally occurring antimicrobials. Edible polymers protect the encapsulated factors from getting affected by the food matrix. It is reported that high concentrations of lipid and carbohydrate can reduce the antimicrobial activity of EOs (de Oliveira et al., 2011). By entrapping the agents in edible polymer, their activity can be prolonged.

Encapsulating the antimicrobial agents could control the interaction between encapsulated antimicrobial and the food matrix. The liquid or small particles could be enclosed with an edible

coating (Quirós-Sauceda et al., 2014), thus protecting them from being integrated by food matrix by the creation of a solid barrier (Quirós-Sauceda et al., 2014).

CHAPTER 2 : ARTICLE-1

FRENCH ABSTRACT

L'activité antimicrobienne des 32 huiles essentielles de plantes (HE) a été évaluée par des méthodes pour inhiber la croissance de quatre bactéries pathogènes d'origine alimentaire (*Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus* et *Salmonella* Typhimurium) et une bactérie d'altération (*Pseudomonas aeruginosa*). Le test de diffusion sur gélose, le dosage de micro-atmosphère et le test de microdilution en bouillon ont été utilisés pour évaluer l'activité antimicrobienne d'HE dans ses phases solides, vapeur et la phase liquide respectivement. À la suite de ces tests, les principaux constituants des HE les plus efficaces (trans-cinnamaldéhyde, carvacrol, thymol, de géraniol et eugénol) ont montré une activité antimicrobienne considérable. Parmi les cinq agents d'origine alimentaire, *Pseudomonas aeruginosa* a montré le moins de sensibilité envers ces HE et *Staphylococcus aureus* s'est avéré être la plus sensible. Pour trouver les interactions antibactériens entre les HE, les meilleurs HE (ayant l'activité antimicrobienne la plus élevée selon leur concentration minimale inhibitrice (CMI) contre chaque bactérie) ont été choisis et l'activité antimicrobienne de mélanges d l'He a été examinés par la méthode de damier. La combinaison de cannelle de Chine et de l'écorce de cannelle a montré des effets antibactériens additifs contre toutes les bactéries cibles. En outre, l'évaluation sensorielle des OE sélectionnés en vigueur dans la viande a également été menée. Il a été perçu qu'une concentration de 0,05% est le seuil maximal d'HE sur la viande cuite pour ce test organoleptique. Une expérience *in situ* a été réalisée avec cette combinaison sur de la viande de porc haché pendant une durée de stockage de 7 jours. L'expérience a prouvé qu'il y avait environ 0,4 – 0,8 log de réduction sur toutes les bactéries testées.

Antimicrobial effect of essential oils in combinations against five bacteria and their effect on sensorial quality of ground meat

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ABSTRACT

The antimicrobial activity of 32 plant Essential Oils (EOs) was assessed by different methods to inhibit the growth of four foodborne pathogenic bacteria (*Escherichia coli, Listeria monocytogenes, Staphylococcus aureus, and, Salmonella* Typhimurium) and one spoilage bacterium (*Pseudomonas aeruginosa*). Agar diffusion, micro-atmosphere and broth microdilution assays were used to evaluate the antimicrobial activity of EOs in solid, vapour and liquid phase, respectively. As a result of these tests, the main constituents of the most effective EOs (trans-cinnamaldehyde, carvacrol, thymol, geraniol, and eugenol) demonstrated considerable antimicrobial activities. Among five foodborne pathogens, *Pseudomonas aeruginosa* showed less and *Staphylococcus aureus* showed the most sensitivity towards these EOs. To find the interactive antibacterial effects among EOs, the EOs with the highest antimicrobial activity based on their Minimum Inhibitory Concentration (MIC) against each bacterium were chosen and antimicrobial activity of combined EOs was examined by the checkerboard method. The combination of Chinese cinnamon and Cinnamon bark showed additive antibacterial effects against all target bacteria. Furthermore, sensory evaluation of selected EOs applied to meat was also conducted. It was perceived that 0.05% is the highest organoleptically acceptable concentration of EOs on cooked meat. *In situ* experiments were performed run with this combination on lean ground pork during storage times of 7 days and it was found that there was approximately 0.4 – 0.8 log reduction among all the tested bacteria.

Key words: Essential oil (EO), food pathogen, spoilage bacteria, interaction of EO, food system

INTRODUCTION

Elimination or inhibition of foodborne pathogenic and spoilage bacteria is highly important for food companies. Food spoilage includes physical damages and/or chemical changes which are due to contamination of food by yeast, mold or bacteria (Cueva et al., 2011; Gutiérrez-Larraínzar et al., 2012). Consumption of contaminated food with pathogenic bacteria causes foodborne illness which is one of the big concerns of public health. According to Thomas et al. (2013), it is estimated that each year in Canada, there are 4.0 million episodes of domestically acquired foodborne illnesses(Thomas et al., 2013).

Food and food products can be contaminated during production, processing, distribution, and preparation (Gaulin et al., 2013). Foodborne pathogens such as *Salmonella* sp., *Staphylococcus aureus, Listeria monocytogenes* and *Escherichia coli* caused the numerous illnesses and death (Oussalah et al., 2007). Also *Pseudomonas aeruginosa* can cause food spoilage and it is one of the reason for off-flavour and discoloration of refrigerated meat (Arslan et al., 2011; Gutierrez et al., 2009). It has been reported that *L. monocytogenes* has been detected in food even after cooking due to post contamination by knifes, containers and, hands among others.

The shelf life, nutrition and microbial quality of food products are important aspects that food companies critically consider. In fact, synthetic preservatives have been widely used to eliminate bacteria and prolong the shelf-life of food products. However, synthetic preservatives may cause environmental and health problems for consumers over a long term period (Jayasena and Jo, 2013; Phillips et al., 2012). Furthermore, consumers nowadays prefer food products with natural preservatives, natural antimicrobial agents and flavors due to their awareness about probable carcinogenic effects of synthetic preservatives and antibiotic resistance after long term usage (Jayasena and Jo, 2013). Thus, searching for new and potential natural antimicrobial agents from

different sources such as microbial metabolites, and plant and spice extracts for food application has been increasing significantly in the past few years (Cueva et al., 2011).

It has been identified that due to co-evolution, plants produce secondary metabolites such as EOs as defense against predators (fungi, insects, etc.). EOs have demonstrated biological properties such as antibacterial, antiparasitic, antifungal, antioxidant and, insecticidal and they have been used since ancient times (Bakkali et al., 2008; Porres-Martínez et al., 2013; Solorzano-Santos and Miranda-Novales, 2012). EOs have an oily consistency and are produced by all organs of plants such as buds, flowers, leaves, stems, seeds, fruits and, roots etc. (Bakkali et al., 2008; Porres-Martínez et al., 2013). Mostly they are extracted from nonwoody organs and are liquid at room temperature (Bassolé and Juliani, 2012; Dorman and Deans, 2000). Over 1340 plants have been identified with antimicrobial compounds (Tajkarimi et al., 2010). According to Burt (2004), out of 3,000 EOs which are already recognized, 300 EOs are commercially important. It is mentioned in several studies that spices and EOs are generally recognized as safe (GRAS) (Burt, 2004; Goñi et al., 2009; Oussalah et al., 2007; Ye et al., 2013). Therefore their antimicrobial properties and their safety make them as one of the best candidates for food companies to use as preservative agents (Goñi et al., 2009).

It has been demonstrated that both major and minor compounds can contribute to the antimicrobial properties of EOs as some minor compounds could have synergetic or additive activity with the major ones or cause the synergy between major compounds (Bassolé and Juliani, 2012; Burt, 2004; Hyldgaard et al., 2012; Oussalah et al., 2007; Turgis et al., 2009). Plant density has an influence on quantitative composition of EOs which, in turn, would determine the properties and activity of EOs (Porres-Martínez et al., 2013). Depending on the climate, region, harvesting season, the parts of the plant used to extract the EOs, as well as the

distillation method used, the EOs may demonstrate different properties in their antimicrobial activity (McGimpsey et al., 1994; Oussalah et al., 2007). For instance according to Rasooli et al. (2006), thyme oils from the top part of plants exhibit strong antimicrobial properties compared with the thyme oils extracted from other parts of plants. So screening the EOs to select the most active one is highly important (Dussault et al., 2014).

Although the EOs are considered as GRAS and have antimicrobial activity, it is necessary to determine their lowest concentration with acceptable antimicrobial activity in order to use them in food without any changes in smell and taste (Turgis et al., 2012). It has been demonstrated that EOs have antimicrobial activity against pathogenic bacteria at the range of 0.05-0.1 % in food systems (Tajkarimi et al., 2010). In fact, the organoleptically acceptable concentration depends on each EO and different food systems, as well as the method of application and cooking methods. Indeed, the antimicrobial activity of EOs may be changed when other compounds are added to the food.

Thus, the main objective of this study was to assess the antimicrobial activities of 32 different EOs against 5 different foodborne pathogens and spoilage bacteria (Gram-negative and Gram-positive) with three methods including agar diffusion assay, micro-atmosphere diffusion assay and microbroth dilution assay. Further, to select the best combined EOs with high antimicrobial effects for food application, the combined effects (synergistic, additive, no interaction, or antagonist effect) of different EOs were evaluated using the checkerboard method. Moreover, sensorial analysis was performed to determine the organoleptically acceptable concentration of selected EOs combination on ground meat as a food model and the antimicrobial efficiency of that concentration was evaluated against target bacteria on meat.

MATERIALS AND METHODS

Preparation of EOs

The list of EOs and their main constituents is presented in Table 1. EOs were prepared as oil-in-water emulsion before utilization for evaluation of their antimicrobial properties. The emulsion of EOs consisted of 2.5 % EO (v/v), 5.0 % Tween 80 (w/v) (Sigma-Aldrich Ltd), (the presence of Tween 80 improved suspension stability), and 92.5 % water (w/w), and was homogenized for 4 minutes at 15000 rpm using an Ultra Turrax (TP18/1059 homogenizer).

Preparation of bacterial cultures

Five bacterial strains, two Gram-positive (*Listeria monocytogenes* HPB 2812 and *Staphylococcus aureus* ATCC 29213) and three Gram-negative (*Escherichia coli* O157:H7 EDL933, *Salmonella* Typhimurium SL 1344 and *Pseudomonas aeruginosa*) were used as target bacteria in antimicrobial tests. With the exception of *P. aeruginosa*, these bacteria were chosen since they represent serious foodborne pathogens. *P. aeruginosa* was chosen as it is one of the leading food spoilage bacteria and it is one of the bacteria that are most resistant to antimicrobial agents. All the bacteria were stored at -80°C in Tryptic Soy Broth (TSB) medium (TSB; BD, Franklin Lakes, NJ, USA) containing glycerol (10% v/v). Prior to each experiment, stock cultures were propagated through two consecutive 24 h growth cycles in TSB at 37°C to reach the concentration of approximately 10^9 CFU/ml and at the same day of experiment, the cultivated cultures were diluted in saline solution to obtain a working culture of approximately 10^6 CFU/ml.

Antimicrobial activity of EOs against target bacteria using agar diffusion assay

The tryptic soy agar (TSA) (Alpha Bioscience) plates were inoculated with a target bacterium. Sterile beads were used to spread 100 µl of a suspension of approximately 10^6 CFU/ml of each bacterium. Then, a sterile 6-mm diameter cellulose test disc was put on the middle of the agar

surface and 4 µl of EO were applied on it. Each plate was closed firmly with Parafilm to prevent vapor transfer from the samples as well the loss of volatile components of EOs (Cardiet et al., 2012; Dussault et al., 2014). Plates were incubated for 72 h at 37°C. The inhibition diameter (colony-free perimeter) around the disc was measured with a Traceable® Carbon Fiber Digital Caliper (resolution: 0.1 mm/0.01''; accuracy: ± 0.2 mm/0.01''; Fisher Scientific).

Evaluation of antimicrobial activity of EOs using micro-atmosphere diffusion assay

To evaluate the antimicrobial activity of volatile compounds of EOs against target bacteria, micro-atmosphere diffusion assay was performed using the inverse Petri dish method (Cardiet et al., 2012). The TSA plates were inoculated with a target bacterium using the same method as mentioned in the agar diffusion assay. The Petri dishes were inverted and different volumes of EOs (10, 20, and, 30 µl) was deposited on a cellulose disc (6mm in diameter) which was placed in the middle of the lid of Petri dish. The Petri dishes were hermetically sealed with Parafilm to prevent vapor transfer between samples as well the loss of volatile components of EOs. Samples were incubated for 24 h at 37°C. The inhibition diameter was measured with a Traceable® Carbon Fiber Digital Caliper.

Determination of minimum inhibitory concentration (MIC) of EOs against target bacteria using broth microdilution assay

The emulsion of each EO was prepared according to modified protocol of Turgis et al. (2012). Serial dilutions were performed from 10000ppm to 10 ppm using Mueller-Hinton (MH) broth. From each concentration, 125 µl of the emulsified EO were taken and filled into wells of column 1 to 11 of a 96-well microplate (Sarstedt, Montreal, QC, Canada). Then, 15 µl of working culture bacteria (approximately 10^6 CFU/ml) were dispensed into all the wells. For each bacterium, three rows of a microplate were used. In the blank or negative control (2 rows of the microplate), 15 µl

of saline solution was used instead of the working culture bacteria. The positive control (without antimicrobial agent) in the column 12 of a microplate consisted of 125 µl of MH broth and 15 µl of working culture bacteria (Turgis et al., 2012). In this test, the final concentration of each EO ranged from 10 ppm to 10000 ppm. The microplate was incubated under aerobic conditions and stirring at 80 rpm for 24 h at 37°C. The absorbance was measured at 595 nm in a BioTek ELx800® absorbance microplate reader (BioTek Instruments Inc., Winooski, VT, USA). The MIC is the lowest concentration of antimicrobial agent demonstrating the complete growth inhibition of the bacterial strain and showing equal absorbance as blank.

Determination of antimicrobial effects of combined EOs using checkerboard method

The checkerboard method was chosen to assess the efficacy of EOs in combination against the pathogens to determine the possible interaction between EOs which could be synergistic, additive, or exhibiting no interaction or antagonist effects. In this method, 96-well microplates were used to obtain the Fractional Inhibitory Concentration (FIC) index of EOs in combinations (Gutierrez et al., 2008, 2009; Turgis et al., 2012). Each of the two selected EOs was two-fold diluted with Mueller-Hinton in two separate microplates. Then the EOs were transferred into the main microplate which contained a serial dilutions of 50 µl of essential oil 'a' (EOa) along the X axis and the serial concentration of same volume of essential oil 'b' (EOb) along the Y axis. In the last 2 rows of the microplate there were only EOa at serial concentrations and in two columns (7 and 8 of the microplate) there were only EOb at serial concentrations. In total, there were a 6 x 6 matrix in which there was a combination of EOa and EOb at different concentrations in each well. Subsequently, 100 µl of Mueller-Hinton (MH) medium containing approximately 2 x 10^6 CFU/ml of one target bacterium were added to the wells. In the last column of the microplate there was MH medium with a target bacterium and was considered as a positive control. The

combinations of EOs without bacteria were filled in other empty columns and were considered as a negative control or blank. Plates were incubated at 37°C for 24 hours with agitation at 80 rpm. The optical density (OD) of the wells containing combined EOs which had same OD of the wells in the blank was used to calculate the FIC. The FIC was calculated by the following formula:

$$FICa = (MICa\ combined\ /\ MICa\ alone)$$

$$FICb = (MICb\ combined\ /\ MICb\ alone)$$

$$FIC = FICa + FICb$$

Where

'MICa alone' is the MIC value of EOa tested alone; 'MICb alone' is the MIC value of EOb tested alone; 'MICa combined' is the MIC value of EOa tested in combination with EOb; 'MICb combined' is the MIC value of EOb tested in combination with EOa.

The results are considered as synergistic when FIC≤0.5, additive when 0.5<FIC≤1, Not interactive for 1<FIC≤4 and antagonistic for the FIC>4.

Sensorial analysis of selected EOs in cooked meat

The most active combination of tested EOs against target bacteria was chosen for the evaluation of their effect on sensorial properties in cooked meat. To find the highest organoleptically accepted concentration, a panel of 10 individuals evaluated the smell and taste of the samples. Several concentrations (0.2, 0.1, 0.05, 0.025 and, 0.0125%) of the selected combination of EOs were applied on ready to cook meat (Kafta) (Adonis, Laval) and also applied on lean ground beef (26% fat) (IGA, Laval, Canada). Both meat models were cooked for 15 minutes at 205°C and when the temperature inside reached 80°C, they were cooked for another 30 seconds. The samples were served in separate cups with closed lids and were identified by 3 random digits.

The evaluation was held on 9-point hedonic scale: 9= Like extremely, 8=Like very much, 7=Like moderately, 6=Like slightly, 5=Neither like nor dislike, 4=Dislike slightly, 3=Dislike moderately, 2=Dislike very much, 1=Dislike extremely

Antimicrobial activity in food system

The most active combination of tested EOs against target bacteria was chosen and the highest organoleptically acceptable concentration of that combination was found through the sensorial analysis mentioned above. Lean ground pork was used as a food system. To sterilize the meat samples before manipulation, they were kept at -80°C under vacuum and were irradiated at 45 kGy. To evaluate the antimicrobial activity of combined EOs in the food system, 4 ml of EOs emulsion (stabilised by Tween 80 and homogenized at 15000 rpm for 4 min) was applied to 20 g of meat and the samples were mixed for 1 minute, then 500, µl of each target bacteria at the concentration of 10^5 CFU/ml was added to the samples and mixed for another 1 minute to obtain a final bacterial concentration of approximately 10^3 CFU/g in ground meat (26% fat). The meat samples were vacuum packed and kept at 4°C. The samples were analysed for the growth of bacteria in day 1, 4, and 7. At each day of analysis, peptone water was added two times more than the samples weight and they were homogenized by a Lab-blender 400 Stomacher (Laboratory Equipment, London, UK) at 230 rpm for 1 minute. Then serial dilutions were performed and all the samples were inoculated in TSA plates for counting. The bacterial colonies were counted after 48 h incubation at 37°C.

Statistical analysis

All the experiments were performed at least two independent times with three replications. One-way analysis of variance (ANOVA) tests using SPSS program (IBM Corporation, Somers, NY,

USA) was conducted to analyze the data. Duncan's multiple range tests was used to compare the mean values. Differences between mean values at $p \leq 0.05$ were considered significant.

RESULTS

Antimicrobial effects of EOs against foodborne and spoilage bacteria in agar diffusion assay

The results of antibacterial effects of 32 EOs against foodborne and spoilage bacteria using the agar diffusion method are presented in Figure 1. The values for the diameter of the inhibition zone (mm) for all tested bacteria are shown by the range of colors from white to black.

The EOs with the inhibition zone less than 10 mm were considered as very low in antibacterial activity, from 10 to 20 mm as bearing low antimicrobial activity, from 20 to 40 as medium antimicrobial activity, from 40 to 60 mm as high antimicrobial activity and finally the EOs with inhibition zones more than 60 mm were considered as very high antimicrobial activity as they could eliminate almost all the bacteria tested on Petri dishes. The results of Figure 1 indicate that, EO of Red thyme, Red bergamot, Ajowan, Winter savory, Chinese cinnamon, and Cinnamon bark demonstrated better inhibitory activity against the five target bacteria as compared with other tested EOs. The diameter of inhibition zone for these oils against tested bacteria was mostly 20 to 40 mm. They showed better results against *S. aureus* (more than 60 mm of inhibition zones for Red thyme, Red bergamot, Ajowan, Winter savory). In addition, these 6 selected oils showed antimicrobial activity (10 to 20 mm for inhibition zone) against *P. aeruginosa* while the other EOs used in this study were unable to inhibit the growth of this bacterium.

Antimicrobial effects of EOs against foodborne and spoilage bacteria in micro-atmosphere assay

The antibacterial effects of 32 EOs against 5 foodborne and spoilage bacteria EOs at 10, 20, and 30 µl using the micro-atmosphere method are presented in Figure 2. It should be mentioned that at the beginning, the experiment was done with a series of volumes of 2, 4, 6, 8, and 10 µl of EOs against all five bacteria; however, it was found that the results were not different (data not shown). Also several EOs could not inhibit the bacteria efficiently at the volume of 10 µl (inhibition zone was less than 10 mm). So, the volume was increased from 10 to 20 and 30 µl in order to determine which concentrations these EOs could inhibit the bacteria efficiently.

As previously seen with the Agar Diffusion Assay, the following descriptors were used: very low antimicrobial activity if inhibition zone <10 mm, low activity if 10 mm <inhibition zone <20 mm, medium activity when 20 mm <inhibition zone <40 mm, high activity, when 40 mm <inhibition zone <60 mm and very high antimicrobial activity for the inhibition zone more than 60 mm. The first two EOs, Chinese cinnamon and Red bergamot were the only EOs which could inhibit all 5 target bacteria. Ajowan, Red thyme, Winter savory and Clove, Oregano, Cinnamon bark, Common thyme generally showed better inhibitory against *L. monocytogenes*, *S. aureus*, *E. coli* and *S.* Typhimurium as compared to other EOs. Some EOs such as Common thyme, Wild bergamot, Chocolate Peppermint, Melissa and Palmarosa were mostly effective with high or very high efficiency against *L. monocytogenes* and *S. aureus*.

Antimicrobial effects of EOs against foodborne and spoilage bacteria in broth microdilution assay

The antibacterial effects of 32 EOs against 5 foodborne and spoilage bacteria in liquid phase (MIC values) are presented in Table 2.

Chinese cinnamon displayed the highest growth inhibitory activity among all the EOs. This EO inhibited the growth of *S. aureus* and *E. coli* at a concentration of 470 ppm (~0.05%) which was the lowest MIC we detected in this study while there were several EOs such as Common juniper, Bay laurel and, Curcuma which were unable to inhibit the bacterial growth even at 10000ppm (~0.1%).

Antibacterial effects of combined EOs against five foodborne and spoilage bacteria using checkerboard method

The antibacterial effects of combined EOs using the checkerboard method against five foodborne and spoilage bacteria are presented in Table 3. This test was conducted to assess the interaction of two EOs in liquid phase in which the most efficient EOs inhibiting each bacterium were chosen according to their MIC values for this test. Red bergamot, Chinese cinnamon, Red thyme, Cinnamon bark, Clove and Wild bergamot were chosen as the most effective EOs in liquid phase against *L. monocytogenes*, *S. aureus*, *E. coli* and *S.* Typhimurium due to their overall lower MIC values against pathogenic bacteria as compared to the other EOs. Among all the tested combinations, the combination of Red bergamot and Clove, Red bergamot and Wild bergamot, Chinese cinnamon and Red thyme showed better efficiency as they showed additive effects against 3 out of 4 tested bacteria. Results showed that the combination of Chinese cinnamon and Cinnamon bark showed additive effect against all tested bacteria. Based on broth microdilution results, Chinese cinnamon, Cinnamon bark and Wild bergamot EOs could also inhibit *P. aeruginosa.* So the combination of Chinese cinnamon with Cinnamon bark and Chinese cinnamon with Wild bergamot were selected to assess their combined antimicrobial effect against *P. aeruginosa* using the checkerboard method and the results were 0.64 ± 0.09 (additive) and 1.29 ± 0.19 (not interactive), respectively. Based on overall results, Chinese cinnamon and

cinnamon bark EOs were selected as a mixture EOs for evaluation of their sensorial and antibacterial effects in a meat model.

Sensorial properties of selected combined EOs in meat products

The results of sensorial analysis are presented in Table 4. The results were the average of scores which the examiners gave to each sample. Based on 9-point hedonic scale the values more than 5 were considered organoleptically acceptable. It was observed that 0.05 % (v/v) of combined EOs (Chinese cinnamon and Cinnamon bark) was acceptable in terms of smell and taste in both ground beef and ready to cook (RTC) ground meat. Thus, this concentration was chosen for the evaluation of the antibacterial effects against 5 target foodborne and spoilage bacteria in the *in situ* experiment.

In situ evaluation

The antibacterial effects of combined EOs against five foodborne and spoilage bacteria on lean ground pork are presented in Table 5. This test was performed to assess the antimicrobial activity of combined EOs in a lean ground pork model against 5 foodborne pathogenic and spoilage bacteria. The combination of EOs (Chinese cinnamon and Cinnamon bark) was selected according to the results of the checkerboard at the final concentration of 0.05% (v/v) as well as the sensorial results. The analysis was performed on day 1, 4 and 7. The log reduction of EOs against each bacterium in each day of analysis was calculated and presented in Table 5.

It is interesting to find that the selected EOs could reduce the growth of all tested bacteria in ground meat at day 1 by at least 0.47 log. Further, this formulation could reduce *E. coli* by 0.8 and 0.5 log at day 1 and 7 days of storage, respectively. The formulation is also active against *S.* Typhimurium.

DISCUSSION

In this study the antimicrobial activity of 32 EOs was evaluated in three phases (liquid, solid and gaseous). The combination effect of EOs was assessed. According to the results EOs such as Red bergamot, Chinese cinnamon, Red thyme, Cinnamon bark, Clove and Wild bergamot generally demonstrated higher antimicrobial activity with all the methods. The interaction between the Chinese cinnamon and Cinnamon bark was additive against all tested bacteria. This combination of EOs was also effective in a meat model at their organoleptic acceptable concentration. EOs are mainly composed of terpenes, terpenoids, and aromatic compounds (Laird and Phillips, 2012). Terpenoids such as geraniol and carvacrol are terpenes which contain oxygen and can be subdivided into alcohols, esters and phenols (Jayasena and Jo, 2013; Solorzano-Santos and Miranda-Novales, 2012). Most of the antimicrobial components of EOs are derived from terpenes. Alcohols and phenolic compounds of EOs are considered as the most effective antimicrobial compounds. For instance, eugenol (83-95% of Clove) and thymol (48.03% of Red thyme, 32.35% of Ajowan, 34.70% of Common thyme and 14.4% of Winter savory) showed antimicrobial activity against *Salmonella* Typhimurium and, *Staphylococcus aureus*. EOs are effective in inhibiting both Gram-negative and Gram-positive microbes, but generally the lipopolysacharide in the outer membrane of Gram-negative bacteria renders them more resistant against EOs than Gram-positive ones (Helander et al., 1998; Jayasena and Jo, 2013; Sivropoulou et al., 1996).

Compared to antibiotics, EOs are volatile compounds at room temperature. A few studies are available in terms of antimicrobial properties of EO in vapour phase and several methods have been used by different authors (Goñi et al., 2009; Lopez et al., 2005; Nedorostova et al., 2009; Tyagi et al., 2012). The disc volatilization test (the one that was used in this study) was the most widely used method. Nevertheless this method has also some disadvantages such as poor sealing,

loss of vapours or having interaction of EOs with the plastic material of the Petri dish cover (Tyagi et al., 2012); however in this study all the experiments were held in the same conditions so that the results were comparable.

EOs need to contact bacteria in order to inhibit their growth or eliminate them, so differences in the antimicrobial effects of EOs in different conditions such as solid, vapour and, liquid phase could be expected (Goñi et al., 2009). The effectiveness of EOs in the vapour phase could be completely different from direct contact in solid and liquid phases (Goñi et al., 2009). In direct contact, hydrophilic components of EOs are more critical than volatile substances in inhibiting the bacteria whereas in the vapour phase the volatile components could be both hydrophilic and hydrophobic (Goñi et al., 2009). Therefore, it could be possible for some EOs to inhibit bacteria more efficiently in the vapour phase rather than the liquid phase depending on the composition of EOs. One of the probable reasons for this difference is the trend of lipophilic molecules to form micelles which decrease the attachment of EOs to microorganisms as compared to vapour phase where there are free attachment (Laird and Phillips, 2012). In this study, carvacrol ($C_6H_3CH_3(OH)(C_3H_7)$ or $C_{10}H_{14}O$, molecular weight (MW) of 152.22 Daltons (Da), thymol ($C_{10}H_{14}O$, an isomeric with carvacrol), are both monoterpenoid phenolic compound; and cinnamaldehyde (C_9H_8O, an aromatic compound, MW of 132.16 Da) were the main compounds in EOs Red bergamot, Chinese cinnamon, Red thyme, Cinnamon bark and these EOs showed high antibacterial activity against different target bacteria.

Based on the data from solid phase micro extraction (SPME) and gas chromatography- mass spectroscopy (GC-MS), monoterpenes are more available in vapour phase as compared to the liquid phase, and the efficiency of EOs in the vapour phase is higher than in the liquid phase where the probability that EOs can attack the bacterial membrane is higher (Tyagi et al., 2012).

So in the vapour phase, EOs could be used at lower concentrations than in solid phase. Indeed they can be used as air decontaminants in storage rooms and they can be good candidates to be used in active packaging. Also due to their volatility they will not change the organoleptic properties of foods (Laird and Phillips, 2012). It is also recommended to verify the interactions among different EOs in vapour phase for possible synergistic effects (Goñi et al., 2009).

In the case of antibacterial activity in solid phase, it is possible to use EOs on the surface of foods to derive benefit of their antimicrobial activity. EOs could be used as coating solution for food products, such as vegetables or fruits which can be dipped or sprayed by the coating solution in order to inhibit the bacteria since many vegetables or fruits could be contaminated with pathogenic bacteria during transporting, storing or packaging. Edible films such as methylcellulose, chitosan, alginate, etc. can be used as carriers for EOs. (Huq et al., 2013; Severino et al., 2014). It is interesting to realize that carvacrol, thymol and cinnamaldehyde were the main compounds of some EOs such as Winter savory, Red bergamot, Chinese cinnamon, Red thyme, and Ajowan which demonstrated medium and high inhibitory activity against four pathogenic bacteria which showed higher antimicrobial activity in vapour phases as well as in solid phase.

Several factors such as temperature, inoculum size, strain and test methods could affect the MIC values. Also it is difficult to monitor the rate of solubility of natural EOs (Ye et al., 2013). In this study, an attempt was made to keep all the experimental conditions identical in order to compare the results. In the liquid phase, Red bergamot, Chinese cinnamon, Cinnamon bark, Clove and, Wild bergamot generally showed high antimicrobial activity against tested bacteria due to their low MIC. As compared with other results, Red bergamot, Chinese cinnamon and Cinnamon bark showed higher antimicrobial activity in solid phases as well. Carvacrol is the major component

of Red bergamot and cinnamaldehyde is the major component of Chinese cinnamon and Cinnamon bark. So it can be inferred, both kinds of compounds (terpenes and phenolic compounds) work very well in solid and liquid phases. Indeed, it can be concluded that eugenol and geraniol, the main components of Clove and Wild bergamot worked better in liquid phase rather than solid phase (low activity).

By comparing all the antibacterial effects of the different performed assays (solid phase, vapour phase and liquid phase), against the tested bacteria in this study, *S. aureus* was the most sensitive bacterium to the tested EOs, followed by *L. monocytogenes*, *E. coli*, *S.* Typhimurium; and finally *P. aeruginosa* was the least sensitive bacteria to selected EOs. Our results are in accordance with previous studies which demonstrated Gram-positive bacteria like *L .monocytogenes* which have been shown to be sensitive to most antimicrobial agents, especially phenolic compounds, rather than Gram-negative bacteria (Gutiérrez-Larraínzar et al., 2012).

EOs are made of several components like menthol, carvacrol, p-cymene, cinnamaldehyde (Burt, 2004), so it is difficult to specify some cellular targets for them; because of their lipophilic characteristic, they can permeabilize the bacterial cells by disrupting the structure of polysaccharides, fatty acids and phospholipids of cell walls and cell membranes. They have cytotoxic properties as they can damage and permeabilize the membrane and therefore, cause the loss of ions and ATP, collapse of proton pumps and finally release of macromolecules and cause bacterial cell lysis (Bakkali et al., 2008; Turgis et al., 2012). By accumulating in the cytoplasm, they could also damage lipids and proteins (Bakkali et al., 2008). The majority of EOs eliminate bacteria by affecting their cell membrane in various ways and cause cell death. For example, tea polyphenols damage the cell membrane of *P. aeruginosa* and inhibit the hemolytic and cholesterol-binding activity of this bacterium (Yi et al., 2010). If two EOs have similar

compositions, it is more probable to show additive effects rather than synergistic effects (Gutierrez et al., 2008). Both Chinese cinnamon and Cinnamon bark possess trans-cinnamaldehyde and they exhibit an additive effect against all tested bacteria.

In this study, by considering the most antibacterial effective EOs and their major components via different methods, it can be deduced that carvacrol, trans-cinnamaldehyde, thymol, eugenol ($C_{10}H_{12}O_2$, MW of 164.2 Da), and also p-cymene ($C_{10}H_{14}$, MW of 134.22 Da) were the main compounds found in the most effective EOs which expressed the highest antimicrobial activities against both Gram-negative and Gram-positive bacteria.

In the case of carvacrol, it is practically immiscible in water. According to Ait-Ouazzou et al. (2013), an acidic pH (4.0) is required for carvacrol to inactivate the bacteria. Carvacrol and thymol are isomeric low-molecular-weight phenolic compounds. They have a non-polar part which can easily go through the bacterial cell membrane and a hydroxyl group that can attach to delocalized electrons which confers an acidic character to these molecules. Besides, H^+ATPase plasma membrane is needed to generate electrochemical proton gradient to keep the homeostasis of internal pH of the cell. It could be the reason of decreasing in ATP concentration and death of cell. ATP should be used to generate electrochemical proton gradient in order to maintain the internal pH in a homeostasis range (Gutiérrez-Larraínzar et al., 2012).

Burt (2004) showed that EOs containing carvacrol have antimicrobial activity against different bacteria, yeast, and fungi (Knowles et al., 2005). Carvacrol is an isoprenyl phenol which has strong antimicrobial activity. It has been shown that carvacrol has a specific effect on *S. aureus*, *S. epidermis* and *L. monocytogenes* which are both Gram-positive bacteria (Ait-Ouazzou et al., 2013; Solorzano-Santos and Miranda-Novales, 2012). The mechanism of carvacrol is similar to

other phenolic compound. Phenolic compounds can kill bacteria by permeabilizing the bacterial cell membrane. So, the bacteria will lose protons and potassium as their membrane is damaged and finally because of losing internal ATP they will die (Kisko and Roller, 2005; Oussalah et al., 2006).

In fact, the cell membrane is the major target of carvacrol. Carvacrol disintegrates outer membrane. and permeabilizes bacteria (Ait-Ouazzou et al., 2013). It also can inhibit *S. aureus* during the early stages of biofilm development which means it is mainly effective against planktonic and nonadherent bacterial cells (Knowles et al., 2005).

Like carvacrol, thymol is mainly effective against Gram-positive bacteria but both of these compounds could disturb the membrane of *E. coli* and *S.* Typhimurium and inhibit these Gram-negative bacteria efficiently (Gutiérrez-Larraínzar et al., 2012; Jirovetz et al., 2006). Thymol is even more effective against *E. coli* than Gram-positive bacteria and it has been showed in our results too as the MIC value of Red thyme for *E. coli* was lower than *L. monocytogenes*. It can be concluded that these two phenolic compounds, carvacrol and thymol are more active than other phenolic compounds such as eugenol, gallic acid etc. (Gutiérrez-Larraínzar et al., 2012).

In the case of eugenol, it is another active antimicrobial compound of EOs and the mechanism of its activity is similar to carvacrol and thymol. As eugenol is liphophilic, it can disturb the cell membrane and cause the loss of chemiosmosis and finally cause the cell death. Compared to carvacrol and thymol, eugenol demonstrated less antimicrobial activity and in our study. It could be explained that because of its molecular structure, eugenol is less hydrophobic than carvacrol and thymol due to having the methoxyl group in ortho position which also makes it unstable in aqueous solutions (Devi et al., 2010; Gutiérrez-Larraínzar et al., 2012).

It can be observed that both Gram-positive and Gram-negative bacteria can be affected by eugenol and cinnamaldehyde. Eugenol showed a better activity against *E. coli* O157:H7 and *L. monocytogenes* while cinnamaldehyde inhibited *S. aureus*, *E. coli* O157:H7, and *S.* Typhimurium efficiently (Gill and Holley, 2004). Ağaoğlu et al. (2007) demonstrated that *P. aeruginosa* and *E. coli* are resistant to may EOs but they were sensitive to cinnamon. Among all five bacteria, *P. aeruginosa* was the most resistant to EOs in all used methods. Also *E. coli* was more resistant compared to *L. monocytogenes* and *S. aureus*. However, the combination of Chinese cinnamon and Cinnamon bark showed an additive effect against this bacteria and the results of *in situ* experiments showed the same trend as the combination of two cinnamons showed 0.82 and 0.62 log reduction against *E. coli* and *P. aeruginosa* respectively.

If an antimicrobial agent could inhibit the energy generation of bacteria, it can kill the bacteria easily as the bacteria cannot produce or change metabolites to adapt itself to new conditions. It has been shown that eugenol and cinnamaldehyde could affect energy generation by inhibiting glucose uptake and permeabilize the bacterial membrane (Gill and Holley, 2004).

For the *in situ* test, lean meat (26% fat) was used in this study. Since high quantity of fat could have affected the bacterial growth by trapping EOs and decreasing the chance of attaching EOs to the bacteria (Gutierrez et al., 2008). Hence the concentration of EOs should be increased in the food with high quantity of fat. Generally in order for the *in situ* experiments to obtain the same inhibitory effect as in the *in vitro* analysis, higher concentrations of EOs are needed because fat, protein and starch protect the bacteria from the action of EOs (Gutierrez et al., 2008). According to our results, the activity of EOs decreased during the storage time. The phenolic or terpene components of the EOs should contact the bacteria to eliminate them. To increase the chance of contacting EOs with the bacteria, 4 ml of EO emulsion was mixed with 20 g of meat. Ground

meat is a complex food system and it is hard to inhibit the growth of bacteria in it due to the high amount of nutrients and moisture, which creates a suitable environment to promote the growth of bacteria. Indeed as it was mentioned in Gutierrez et al. (2008), the activity of EOs decreased when added to a complex food system. In accordance with the results of MIC, as both EOs Chinese cinnamon and Cinnamon bark showed the lowest MIC against *E. coli*, in the *in situ* test, the best results were those obtained with *E. coli* which reduced by 0.8 and 0.5 log after one and 7 days of storage, respectively. Also Chinese cinnamon had low MIC against *S. aureus* which could explain the reason of the growth inhibition by around 0.8 log of bacteria after one day of storage. Indeed these results are in accordance with Gill et al. (2004) which demonstrated cinnamaldehyde inhibited *S. aureus*, *E. coli* O157:H7, and *S.* Typhimurium efficiently.

CONCLUSION

In this study, Red thyme, Red bergamot, Winter savory, Chinese cinnamon and Cinnamon bark overall showed high activity against all the tested bacteria. Also additive effects occurred with a number of combinations of EOs. Specifically Chinese cinnamon and Cinnamon bark showed high efficiency and additive effect against pathogenic and spoilage bacteria. At their organoleptically acceptable concentration (0.05%) this combination of EOs inhibited the growth of target bacteria in our food system. So according to our results, this combination of EO could have high potential for the preservation of meat products.

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References

1. Ağaoğlu, S., Dostbil, N., Alemdar, S., 2007. Antimicrobial activity of some spices used in the meat industry. Bull Vet Inst Pulawy 51, 53-57.
2. Ait-Ouazzou, A., Espina, L., Gelaw, T.K., de Lamo-Castellvi, S., Pagan, R., Garcia-Gonzalo, D., 2013. New insights in mechanisms of bacterial inactivation by carvacrol. Journal of Applied Microbiology 114, 173-185.
3. Arslan, S., Eyi, A., Özdemir, F., 2011. Spoilage potentials and antimicrobial resistance of *Pseudomonas* spp. isolated from cheeses. Journal of dairy science 94, 5851-5856.
4. Bakkali, F., Averbeck, S., Averbeck, D., Idaomar, M., 2008. Biological effects of essential oils – A review. Food and Chemical Toxicology 46, 446-475.
5. Bassolé, I.H.N., Juliani, H.R., 2012. Essential Oils in Combination and Their Antimicrobial Properties. Molecules 17, 3989-4006.
6. Burt, S., 2004. Essential oils: their antibacterial properties and potential applications in foods—a review. International Journal of Food Microbiology 94, 223-253.
7. Cardiet, G., Fuzeau, B., Barreau, C., Fleurat-Lessard, F., 2012. Contact and fumigant toxicity of some essential oil constituents against a grain insect pest Sitophilus oryzae and two fungi, Aspergillus westerdijkiae and Fusarium graminearum. Journal of Pest Science 85, 351-358.
8. Cueva, C., Moreno-Arribas, M.V., Bartolome, B., Salazar, O., Vicente, M.F., Bills, G.F., 2011. Antibiosis of vineyard ecosystem fungi against food-borne microorganisms. Research in Microbiology 162, 1043-1051.
9. Devi, K.P., Nisha, S.A., Sakthivel, R., Pandian, S.K., 2010. Eugenol (an essential oil of clove) acts as an antibacterial agent against Salmonella typhi by disrupting the cellular membrane. Journal of Ethnopharmacology 130, 107-115.

10. Dorman, H.J., Deans, S.G., 2000. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. Journal of Applied Microbiology 88, 308-316.
11. Dussault, D., Vu, K.D., Lacroix, M., 2014. In vitro evaluation of antimicrobial activities of various commercial essential oils, oleoresin and pure compounds against food pathogens and application in ham. Meat Science 96, 514-520.
12. Gaulin, C., Nguon, S., Leblanc, M.A., Ramsay, D., Roy, S., 2013. Multiple outbreaks of gastroenteritis that were associated with 16 funerals and a unique caterer and spanned 6 days, 2011, Quebec, Canada. Journal of Food Protection 76, 1582-1589.
13. Gill, A.O., Holley, R.A., 2004. Mechanisms of bactericidal action of cinnamaldehyde against *Listeria monocytogenes* and of eugenol against *L. monocytogenes* and Lactobacillus sakei. Applied and Environtal Microbiology 70, 5750-5755.
14. Goñi, P., López, P., Sánchez, C., Gómez-Lus, R., Becerril, R., Nerín, C., 2009. Antimicrobial activity in the vapour phase of a combination of cinnamon and clove essential oils. Food Chemistry 116, 982-989.
15. Gutiérrez-Larraínzar, M., Rúa, J., Caro, I., de Castro, C., de Arriaga, D., García-Armesto, M.R., del Valle, P., 2012. Evaluation of antimicrobial and antioxidant activities of natural phenolic compounds against foodborne pathogens and spoilage bacteria. Food Control 26, 555-563.
16. Gutierrez, J., Barry-Ryan, C., Bourke, P., 2008. The antimicrobial efficacy of plant essential oil combinations and interactions with food ingredients. International Journal of Food Microbiology 124, 91-97.

17. Gutierrez, J., Barry-Ryan, C., Bourke, P., 2009. Antimicrobial activity of plant essential oils using food model media: Efficacy, synergistic potential and interactions with food components. Food Microbiology 26, 142-150.
18. Helander, I.M., Alakomi, H.L., Latva-Kala, K., Mattila-Sandholm, T., Pol, I., Smid, E.J., Gorris, L.G.M., Wright, A., 1998. Characterization of the action of selected essential oil components on gram-negative bacteria. Journal of agricultural and food chemistry 46, 3590-3595.
19. Huq, T., Khan, A., Khan, R.A., Riedl, B., Lacroix, M., 2013. Encapsulation of probiotic bacteria in biopolymeric system. Crit Rev Food Sci Nutr 53, 909-916.
20. Hyldgaard, M., Mygind, T., Meyer, R.L., 2012. Essential oils in food preservation: mode of action, synergies, and interactions with food matrix components. Frontiers in Microbiology 3.
21. Jayasena, D.D., Jo, C., 2013. Essential oils as potential antimicrobial agents in meat and meat products: A review. Trends in Food Science & Technology 34, 96-108.
22. Jirovetz, L., Buchbauer, G., Stoilova, I., Stoyanova, A., Krastanov, A., Schmidt, E., 2006. Chemical Composition and Antioxidant Properties of Clove Leaf Essential Oil. Journal of agricultural and food chemistry 54, 6303-6307.
23. Kisko, G., Roller, S., 2005. Carvacrol and p-cymene inactivate Escherichia coli O157:H7 in apple juice. BMC Microbiol 5, 36.
24. Knowles, J.R., Roller, S., Murray, D.B., Naidu, A.S., 2005. Antimicrobial action of carvacrol at different stages of dual-species biofilm development by *Staphylococcus aureus* and Salmonella enterica serovar Typhimurium. Applied and Environmental Microbiology 71, 797-803.

25. Laird, K., Phillips, C., 2012. Vapour phase: a potential future use for essential oils as antimicrobials? Letters in Applied Microbiology 54, 169-174.
26. Lopez, P., Sanchez, C., Batlle, R., Nerin, C., 2005. Solid-and vapor-phase antimicrobial activities of six essential oils: susceptibility of selected foodborne bacterial and fungal strains. Journal of agricultural and food chemistry 53, 6939-6946.
27. McGimpsey, J.A., Douglas, M.H., Van Klink, J.W., Beauregard, D.A., Perry, N.B., 1994. Seasonal variation in essential oil yield and composition from naturalized thymus vulgaris L. in New Zealand. Flavour and Fragrance Journal 9, 347-352.
28. Nedorostova, L., Kloucek, P., Kokoska, L., Stolcova, M., Pulkrabek, J., 2009. Antimicrobial properties of selected essential oils in vapour phase against foodborne bacteria. Food Control 20, 157-160.
29. Oussalah, M., Caillet, S., Lacroix, M., 2006. Mechanism of action of Spanish oregano, Chinese cinnamon, and savory essential oils against cell membranes and walls of Escherichia coli O157:H7 and *Listeria monocytogenes*. Journal of Food Protection 69, 1046-1055.
30. Oussalah, M., Caillet, S., Saucier, L., Lacroix, M., 2007. Inhibitory effects of selected plant essential oils on the growth of four pathogenic bacteria: *E. coli* O157:H7, *Salmonella* Typhimurium, *Staphylococcus aureus* and *Listeria monocytogenes*. Food Control 18, 414-420.
31. Phillips, C.A., Laird, K., Allen, S.C., 2012. The use of Citri-V™® — An antimicrobial citrus essential oil vapour for the control of Penicillium chrysogenum, Aspergillus niger and Alternaria alternata in vitro and on food. Food Research International 47, 310-314.

32. Porres-Martínez, M., González-Burgos, E., Accame, M.E.C., Gómez-Serranillos, M.P., 2013. Phytochemical composition, antioxidant and cytoprotective activities of essential oil of Salvia lavandulifolia Vahl. Food Research International 54, 523-531.
33. Severino, R., Vu, K.D., Donsì, F., Salmieri, S., Ferrari, G., Lacroix, M., 2014. Antimicrobial effects of different combined non-thermal treatments against Listeria monocytogenes in broccoli florets. Journal of Food Engineering 124, 1-10.
34. Sivropoulou, A., Papanikolaou, E., Nikolaou, C., Kokkini, S., Lanaras, T., Arsenakis, M., 1996. Antimicrobial and Cytotoxic Activities of Origanum Essential Oils. Journal of agricultural and food chemistry 44, 1202-1205.
35. Solorzano-Santos, F., Miranda-Novales, M.G., 2012. Essential oils from aromatic herbs as antimicrobial agents. Curr Opin Biotechnol 23, 136-141.
36. Tajkarimi, M.M., Ibrahim, S.A., Cliver, D.O., 2010. Antimicrobial herb and spice compounds in food. Food Control 21, 1199-1218.
37. Thomas, M.K., Murray, R., Flockhart, L., Pintar, K., Pollari, F., Fazil, A., Nesbitt, A., Marshall, B., 2013. Estimates of the burden of foodborne illness in Canada for 30 specified pathogens and unspecified agents, circa 2006. Foodborne Pathog Dis 10, 639-648.
38. Turgis, M., Han, J., Caillet, S., Lacroix, M., 2009. Antimicrobial activity of mustard essential oil against Escherichia coli O157:H7 and Salmonella typhi. Food Control 20, 1073-1079.
39. Turgis, M., Vu, K.D., Dupont, C., Lacroix, M., 2012. Combined antimicrobial effect of essential oils and bacteriocins against foodborne pathogens and food spoilage bacteria. Food Research International 48, 696-702.

40. Tyagi, A.K., Malik, A., Gottardi, D., Guerzoni, M.E., 2012. Essential oil vapour and negative air ions: A novel tool for food preservation. Trends in Food Science & Technology 26, 99-113.
41. Ye, H., Shen, S., Xu, J., Lin, S., Yuan, Y., Jones, G.S., 2013. Synergistic interactions of cinnamaldehyde in combination with carvacrol against food-borne bacteria. Food Control 34, 619-623.
42. Yi, S.M., Zhu, J.L., Fu, L.L., Li, J.R., 2010. Tea polyphenols inhibit Pseudomonas aeruginosa through damage to the cell membrane. International Journal of Food Microbiology 144, 111-117.

Table 1. Selected essential oils for evaluation their antimicrobial activity against five pathogens

#	Latin name	Common name	Origin	Distilled part	Composition (%)[2]
1	*Melissa officinalis*	Melissa	Quebec	Aerial part	β-Caryophyllene (23.31), geranial (11.58), germacrene-D (11.49), neral (6.76), geraniol (6.01)
2	*Juniperus communis*	Common juniper	Quebec	Twigs-Berries	α-Pinene (75.61), δ-3-carene (5.46), β-pinene (3.88), myrcene (3.18)
3	*Mentha piperita choco.*	Chocolate Peppermint	Quebec	Aerial part	Menthol (40.12), mentone (25.29), 1,8-cineole (5.90)
4	*Cuminum cyminum*	Cumin	Egypt	Seeds	Cuminic aldehyde (39.16), β-pinene (15.69), ɣ-terpinene (15.63), *p*-cymene (12.47), *p*-menthadiene (8.11)
5	*Satureja hortensis*	Winter savory	Hungary	Flower top	Carvacrol (26.8), *p*-cymene (23.6), thymol (14.4)
6	*Laurus nobilis*	Bay laurel	Hungary	Leaves	1,8-Cineole (48.1), α-terpineol (6.8), sabinene (6.4), α-pinene (6.3), β-pinene (5.8)
7	*Monarda didyma*	Red bergamot	Quebec	Flower top	Carvacrol (48.21), *p*-cymene (13.98), ɣ-terpinene (12.69)
8	*Curcuma longa*	Curcuma	Madagascar	Root	β-Turmerone (36.14), α-turmerone (28.60), myrcene + α-phellandrene (7.90)
9	*Cinnamomum cassia*	Chinese cinnamon	Vietnam	Bark	*Trans*-cinnamaldehyde (87.58), cinnamyl acetate (7.53)
10	*Rosmarinus officinalis*	Rosemary	Morocco	Aerial part	1,8-Cineole (44.48), α-pinene (12.45), camphor (10.70)
11	*Ledum groenlandicum*	Labrador tea	Quebec	Flower top	Limonene (9.94), sabinene (4.72), 1,4-terpinenol (4.36), β-pinene (3.95), mirtenal

					(3.83), α-pinene (3.60)
12	*Thymus vulgaris*	Common thyme	Bolivia	Aerial part	Thymol (34.70), ɣ-terpinene (19.87), *p*-cymene (19.47)
13	*Thymus zygis*	Red thyme	Spain	Aerial part	Thymol (48.03), *p*-cymene (16.60), ɣ-terpinene (8.18)
14	*Origanum kaliteria*	Oregano	Bolivia	Aerial part	Carvacrol (21.01), 1,4-terpinenol (18.68), 4-thujanol (12.14)
15	*Cinnamomum verum*	Cinnamon bark	Madagascar	Bark	*Trans*-cinnamaldehyde (40.71), cinnamyl acetate (14.25), β-phellandrene (9.02), β-caryophyllene (7.41)
16	*Melaleuca quinquenervia cineolifera*	Niaouli	Madagascar	Leaves	1,8-Cineole (53.39), α-pinene + α-thujene (9.16), limonene (7.93), α-terpineol + terpenyl acetate (7.81)
17	*Salvia officinalis*	Common sage	Spain	Aerial part	α-Thujone (35.37), camphor (11.05), 1,8-cineole (8.31), β-thujone (6.93)
18	*Eugenia caryophyllus*	Clove	Madagascar	Floral buds	Eugenol (83-95), eugenyl acetate (9.96), β-caryophyllene (4.01)
19	*Tsuga canadensis*	Hemlock spruce	Quebec	Branches-Needles	Bornyl acetate (38.44), α-pinene (17.64), camphene (14.05)
20	*Monarda fistulosa*	Wild bergamot	France	Flower top	Geraniol (91.71)
21	*Cymbopogon martinii*	Palmarosa	India	Aerial part	Geraniol (80.14), geranyl acetate (9.10)
22	*Cinnamomum camphora*	Ravintsara	Madagascar	Leaves	1,8-Cineole (57.13), sabinene (14.46), α-terpineol (8.76)
23	*Abies balsamea*	Balsam fir	Quebec	Needles	β-Pinene (31.41), δ-3-carene (15.47), α-pinene (13.00), bornyl acetate (9.20), limonene (8.40)
24	*Thuja occidentalis*	Eastern white cedar	Quebec	Branches	α-Thujone (45.65), fenchone (12.11), β-thujone (8.37), sabinene (4.00)
25	*Picea mariana*	Black spruce	Quebec	Branches-	Bornyl acetate (31.01),

				Needles	camphene (18.16), α-pinene (14.00), δ-3-carene (6.42)
26	*Picea glauca*	White spruce	Quebec	Branches-Needles	Bornyl acetate (17.81), β-pinene (13.77), camphor (13.26), α-pinene (12.16), camphene (11.47), β-phellandrene (11.28)
27	*Solidago canadensis*	Canada Golden-rod	Quebec	Flower top	D-Germacrene (28.59), α-pinene (15.90), limonene (12.65), myrcene (7.92)
28	*Daucus carota*	Wild carrot	Quebec	Seeds	Sabinene (31.72), geranyl acetate (15.23), α-pinene (14.58), myrcene (4.90)
29	*Pinus resinosa*	Red pine	Quebec	Twigs-Buds	α-Pinene (49.49), β-pinene (32.26), myrcene (5.90)
30	*Pinus strobus*	White pine	Quebec	Twigs-Buds	α-Pinene (29.82), β-pinene (26.60), δ-3-carene (9.62), myrcene (8.59), limonene (8.52)
31	*Pinus sylvestris*	Scots pine	Quebec	Twigs-Buds	δ-3-carene (30.52), α-pinene (26.91), limonene (7.37), β-pinene (5.78)
32	*Trachyspermum ammi*	Ajowan	India	Seeds	ɣ-terpinene (36.40), thymol (32.35), *p*-cymene (24.72), camphene (2.71)

[1]Essential oils were provided by Aliksir Inc. (Grondines, QC, Canada). [2]Composition determined by gas chromatography analysis using 2 capillary columns (30 m × 0.25 mm): Supelcowax 10 (polar) and DB-5 (apolar). Composition determination was done by Aliksir Inc.

Table 2. Minimum inhibitory concentration (MIC) of EOs against *L. monocytogenes*, *S. aureus*, *E. coli*, *S.* Typhimurium and *P. aeruginosa*

# EO		MIC (ppm)				
		Gram(+) bacteria		Gram(-) bacteria		
	Common Name	*L. monocytogenes*	*S. aureus*	*E. coli*	*S.* Typhimurium	*P. aeruginosa*
1	Melissa	3125	5000	-[1]	-	-
2	Common juniper	-	-	-	-	-
3	Chocolate Peppermint	1880	7500	8750	4375	-
4	Cumin	1250	5000	10000	-	-
5	Winter savory	5000	2500	5000	3130/5000	-
6	Bay laurel	-	-	-	-	-
7	Red bergamot	1250	2500	1250	5000	-
8	Curcuma	-	-	-	-	-
9	Chinese cinnamon	625	470	470	940	1250
10	Rosemary	4380	-	-	10000	-
11	Labrador tea	-	-	-	-	-
12	Common thyme	-	-	-	-	-
13	Red thyme	10000	1250	1250	-	-
14	Oregano	-	6250	5000	3330	-
15	Cinnamon bark	780	1250	780	1250	2500
16	Niaouli	3880	10000	-	-	-
17	Common sage	-	-	-	-	-
18	Clove	3750	1875	1875	3750	-
19	Hemlock spruce	-	-	-	-	-
20	Wild bergamot	3125	1875	1250	1875	10000
21	Palmarosa	3750	7500	5000	5000	-

22	**Ravintsara**	10000	-	-	-	-
23	**Balsam fir**	-	-	-	-	-
24	**Eastern white cedar**	-	10000	-	-	-
25	**Black spruce**	-	-	-	-	-
26	**White spruce**	-	-	-	-	-
27	**Canada Golden-rod**	-	-	-	-	-
28	**Wild carrot**	10000	-	-	-	-
29	**Red pine**	10000	-	-	-	-
30	**White pine**	10000	-	-	-	-
31	**Scots pine**	-	-	-	-	-
32	**Ajowan**	5000	3750	5000	-	-

[1]**(-)**: MIC > 10000 ppm.

Table 3. Fractional Inhibitory Concentration (FIC) of combined EOs against target bacteria

	L. monocytogenes	Act[1]	*S. aureus*	Act	*E. coli*	Act	*S.* Typhimurium	Act
Red bergamot + Chinese cinnamon	0.66 ± 0.13	AD	0.67 ± 0.12	AD	1.06 ± 0.00	I	1.12 ± 0.10	I
Red bergamot + Red thyme	1.12 ± 0.10	I	0.59 ± 0.05	AD	1.07 ± 0.04	I	0.76± 0.25	AD
Red bergamot + Cinnamon bark	1.12 ± 0.11	I	1.11 ± 0.11	I	1.11 ± 0.11	I	1.03 ± 0.02	I
Red bergamot + Clove	0.91 ± 0.15	AD	1.26 ± 0.22	I	0.51 ± 0.03	AD	0.63 ± 0.10	AD
Red bergamot + Wild bergamot	0.63 ± 0.10	AD	0.64 ± 0.09	AD	1.14 ± 0.09	I	0.59 ± 0.05	AD
Chinese cinnamon + Red thyme	1.05 ± 0.06	I	0.47 ± 0.09	AD	0.74 ± 0.00	AD	0.53 ± 0.02	AD
Chinese cinnamon + Cinnamon bark	0.64 ± 0.09	AD	0.60 ± 0.03	AD	0.74 ± 0.00	AD	0.82 ± 0.00	AD
Chinese cinnamon + Clove	1.08 ± 0.03	I	1.10 ± 0.03	I	0.70 ± 0.06	AD	1.20 ± 0.06	I
Chinese cinnamon + Wild bergamot	1.14 ± 0.09	I	0.70 ± 0.0	AD	1.12 ± 0.12	I	0.82 ± 0.14	AD
Red thyme + Cinnamon bark	1.07 ± 0.04	I	1.04 ± 0.01	I	0.64 ± 0.09	AD	1.14 ± 0.09	I
Red thyme + Clove	0.94 ± 0.17	AD	1.22 ± 0.23	I	1.12 ± 0.13	I	0.94 ± 0.17	AD
Cinnamon bark + Clove	1.05 ± 0.05	I	1.09 ± 0.05	I	1.08 ± 0.03	I	1.08 ± 0.03	I
Cinnamon bark + Wild bergamot	1.06 ± 0.05	I	0.64 ± 0.09	AD	1.04 ± 0.01	I	1.08 ± 0.03	I

[1]Act = Activity: FIC≤0.5: synergic effect (S); 0.5<FIC≤1: additive effect (AD); 1<FIC≤4: no interactive effect (I); FIC>4: antagonistic effect (A)

Table 4. Sensorial evaluation of two kinds of meat with a series concentration of combined EOs (Chinese cinnamon and Cinnamon bark)

Properties	**Smell**		**Taste**	
	RTC[1] meat	**Ground beef**	**RTC meat**	**Ground beef**
Control	6.40 ± 1.95^{b2}	6.75 ± 1.28^{bc}	6.88 ± 2.02^{c}	6.66 ± 1.75^{c}
0.0125%	6.00 ± 1.94^{b}	5.37 ± 0.91^{bc}	6.33 ± 1.11^{bc}	5.16 ± 2.48^{bc}
0.025%	6.40 ± 1.42^{b}	5.00 ± 1.69^{abc}	6.33 ± 2.34^{bc}	5.83 ± 1.60^{c}
0.05%	6.00 ± 2.05^{b}	5.25 ± 2.12^{bc}	5.88 ± 2.47^{bc}	5.00 ± 2.00^{bc}
0.1%	5.60 ± 2.50^{b}	3.75 ± 1.75^{ab}	4.55 ± 2.24^{ab}	3.50 ± 1.51^{ab}
0.2%	3.00 ± 1.41^{a}	3.37 ± 1.59^{a}	3.22 ± 2.33^{a}	2.33 ± 1.21^{a}

[1]RTC = ready-to-cook. [2] In the same column bearing the same lower case letters are not significantly different ($p > 0.05$).

Table 5. The log reduction (CFU/g) of combination of Chinese cinnamon and Cinnamon bark EOs against 5 target bacteria during storage time on lean ground pork

	Day 1	**Day 4**	**Day 7**
Bacteria	Log reduction (CFU/g)	Log reduction (CFU/g)	Log reduction (CFU/g)
L. monocytogenes	0.47 ± 0.21^{bA1}	0.34 ± 0.04^{abA}	0.16 ± 0.04^{aA}
S. aureus	0.85 ± 0.06^{bB}	0.47 ± 0.13^{aAB}	0.23 ± 0.22^{aAB}
E. coli	0.82 ± 0.19^{bB}	0.72 ± 0.12^{abB}	0.52 ± 0.19^{aB}
S. Typhimurium	0.67 ± 0.17^{aAB}	0.58 ± 0.26^{aAB}	0.40 ± 0.22^{aAB}
P. aeruginosa	0.62 ± 0.20^{bAB}	0.33 ± 0.19^{abA}	0.23 ± 0.22^{aAB}

[1]In the same column bearing the same upper case letters and in the same row bearing the same lower case letters are not significantly different ($p > 0.05$).

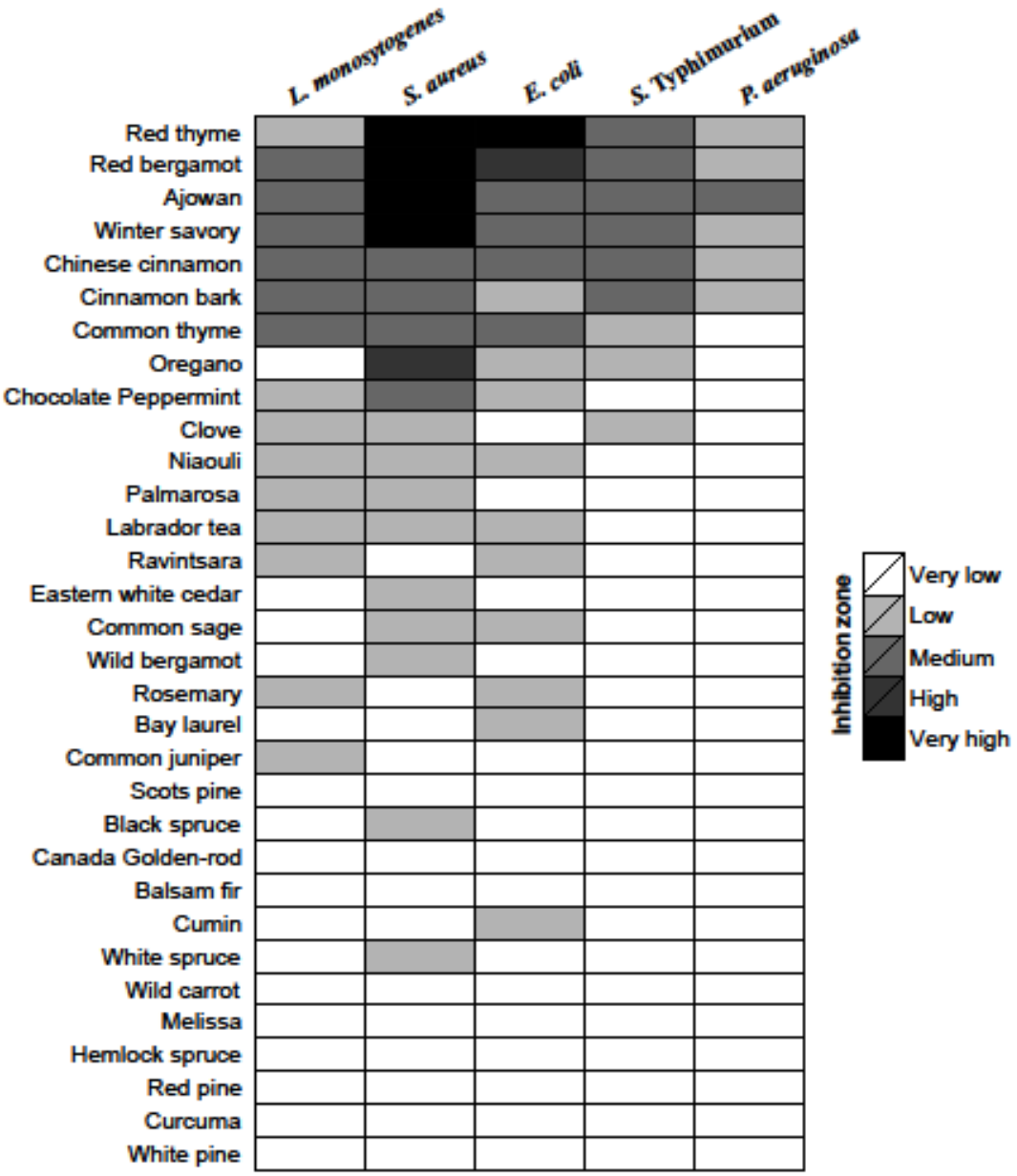

Figure 1. Antibacterial effects of EOs against five foodborne and spoilage bacterium in agar diffusion assay

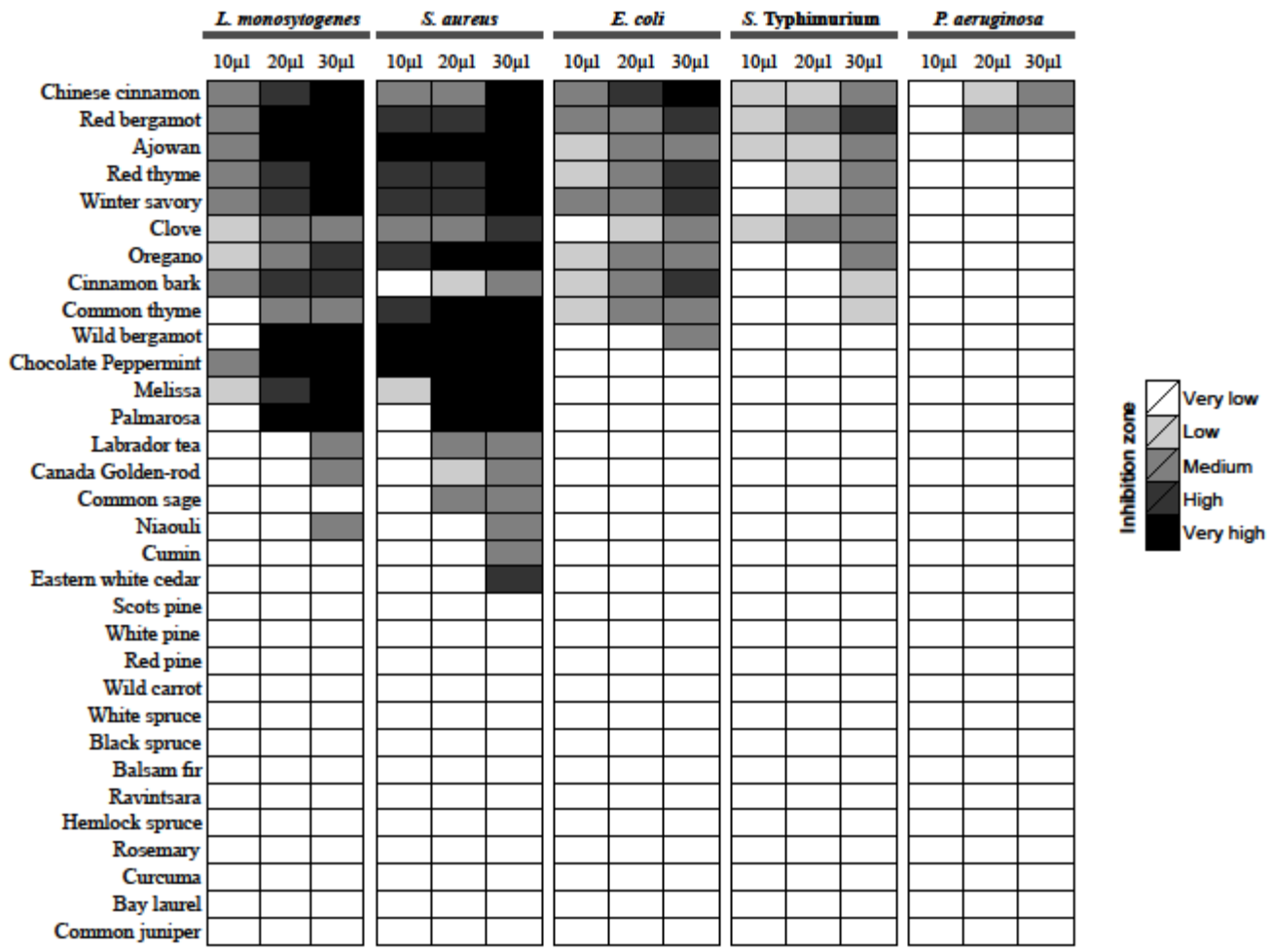

Figure 2. Antibacterial effects of EOs against five foodborne and spoilage bacterium in micro-atmosphere assay at 10, 20 and 30µl.

CHAPTER 3 : ARTICLE-2

FRENCH ABSTRACT

Cette étude a été menée pour évaluer les activités antimicrobiennes de seize formulations contre *Listeria* dans un modèle de saucisse. Pour développer une technologie multi variable à contrôler efficacement la bactérie *Listeria monocytogenes*, un design expérimental standard avec 4 facteurs indépendants à 2 niveaux (4^2) a été mené. Quatre facteurs indépendants consistaient eu un mélange des huiles essentielles (HE) de cannelle de Chine et de l'écorce de cannelle (qui ont été choisis en fonction de leur activité antimicrobienne selon nos études), de nisine, de nitrites et de sels d'acides organiques. Le haut niveau est la valeur maximale de la concentration autorisée ou acceptable de chaque facteur et le faible niveau est la moitié de la valeur du haut niveau de chaque facteur. Basé sur l'analyse, l'utilisation de 0,025 ou 0,05% d'HE en combinaison avec de faibles concentrations de nitrite (100 ppm), de sels d'acides organiques (1,54%), et de nisine (12,5 ppm) pourrait réduire respectivement 1,5 ou 2,6 log UFC / g de *L. monocytogenes* dans les saucisses au jour 7 de stockage par rapport à la commande. L'évaluation sensorielle, puis a ensuite été réalisée sur une sélection de formulations optimisées dans la viande cuite (à la fois le porc et les saucisses de bœuf) avec un jury composé de 35 personnes, a démontré que les meilleures formulations antimicrobiennes sont également du point de vue organoleptique acceptables.

Optimization of antibacterial activity of sixteen formulations containing essential oils, nisin, nitrite and organic acid salts against *Listeria monocytogenes* in a sausage model

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ABSTRACT

This study was conducted to evaluate the antimicrobial activities of sixteen formulations against Listeria in a sausage model. To develop a Hurdle technology to control effectively *Listeria monocytogenes*, a standard experimental design with 4 independent factors at 2 levels (4^2) was conducted. Four independent factors consisted of the mixture of Chinese cinnamon and Cinnamon bark Essential Oils (EOs) which were chosen based on their antimicrobial activity according to our previous study, nisin, nitrite and organic acid salts. The high level is the maximum value of permitted or acceptable concentration of each factor and the low level is the half value of the high level of each factor. Based on the analysis, utilization of 0.025 or 0.05 % EOs in combination with low concentrations of nitrite (100 ppm), organic acid salts (1.54%), and nisin (12.5 ppm) could reduce respectively 1.5 or 2.6 log CFU/g of *L. monocytogenes* in sausage at day 7 of storage as compared to the control. The sensory evaluation was then performed on selected optimized formulation in cooked meat (both pork and beef sausages) with a trained jury consisting of 35 individuals, demonstrated the best antimicrobial formulations are also organoleptically acceptable.

Key words: *Listeria monocytogenes*, essential oil, Hurdle technology, sausage, organoleptic properties

INTRODUCTION

Each year, contaminated food products cause numerous foodborne diseases. As for instance, in the United States, foodborne diseases cause 9.4 million illnesses, 55,961 hospitalisations and 1,391 deaths each year (Scallan et al., 2011). In Canada there are approximately 4 million foodborne illnesses which cause an economic burden of approximately $3.7 billion annually (Nesbitt et al., 2014). Food products can get contaminated during preparation, storage and distribution so it is important that they are well protected against foodborne pathogens (Fratianni et al., 2010).

Meat and meat products, due to their high level of bacterial nutrients, pH (5.5-7.0) and high water activity, offer a congenial environment for the growth of bacteria, (Dave and Ghaly, 2011). Hence meat and meat products should be handled and preserved properly otherwise they are susceptible to the growth of pathogenic and spoilage bacteria. Among all the food pathogens, *L. monocytogenes* is one of the most dangerous types. It is a Gram-positive bacterium, facultative anaerobe pathogen and causes listeriosis which is a severe human disease associated with meningitis and gastroenteritis (Solomakos et al., 2008b). It was reported that *L. monocytogenes* causes 19% foodborne illnesses in USA annually (Scallan et al., 2011). The major risk factor for *L. monocytogenes* infection is by consuming undercooked ground beef (Solomakos et al., 2008b). Meat products can be contaminated by *L. monocytogenes* during preparation, storage and distribution (Fratianni et al., 2010). Further, the important point is that *L. monocytogenes* can grow at refrigerated conditions which make it more dangerous and difficult to control. The contamination of *L. monocytogenes* can also be due to inadequate temperature for cooking or post contamination with contaminated hands, knives or other dishes (Ramaswamy et al., 2007; Samaxa et al., 2012).

Nowadays, consumers concerned about probable toxic and carcinogenic effects of synthetic antimicrobial agents used in food products. They are also concerned about resistance of pathogenic bacteria to these synthetic agents, and prefer food products using natural antimicrobial agents (Jayasena and Jo, 2013). Thus food companies are now readily interested to use natural food additives to replace completely or partially synthetic additives. Herbal extracts or Essential Oils (EOs) are among of the natural compounds that can be used in food products due to their GRAS (Generally Recognized As Safe) status (Oussalah et al., 2007) and their antimicrobial efficacy against several foodborne pathogens, especially *L. monocytogenes* (Burt, 2004; Fratianni et al., 2010). Many studies have showed the antimicrobial effect of EOs against variety of pathogenic and spoilage microorganisms. It has been found some EOs such as oregano, rosemary, thyme, clove, balm, ginger, basilica, coriander, marjoram, and basil demonstrated high antimicrobial efficacy on meat and meat products (Jayasena and Jo, 2013).

EOs are aromatic and organic liquids extracted from plants, which have been traditionally used as aroma and spices in food. The European Commission and the United States Food and Drug Administration (FDA) accepted some EOs constituents like cinnamaldehyde, thymol, eugenol, carvacrol as well as crude EOs such as cinnamon, mustard, oregano, thyme, clove and so forth to be used in food products (Hyldgaard et al., 2012). It has been known that EOs at high concentration may cause sensorial side effects on food products (Abdollahzadeh et al., 2014; Burt, 2004). In order to enhance their antimicrobial efficacy, EOs are widely using in combination with other antimicrobial compounds. Some studies revealed synergetic interaction between EOs and other antimicrobial components in meat applications (Solomakos et al., 2008a, b).

Nisin is a ribosomally synthesized cationic polypeptide having antimicrobial activity and is one of the most popular bacteriocins. It is a heat-stable compound produced by Lactic Acid Bacteria (LAB) and has GRAS status. It is approved and widely used in more than 50 countries (Abdollahzadeh et al., 2014). Nisin has been found as a candidate antimicrobial compound against *L. monocytogenes* for meat applications (Abdollahzadeh et al., 2014; Millette et al., 2007; Solomakos et al., 2008b). Potassium lactate (PL) and sodium acetate (SA) are antimicrobial agents which are recognized as safe (GRAS). These organic acid salts are widely used as food preservatives (Perumalla et al., 2012). Nitrate (NO_3) has been used since 19^{th} century as a food preservative especially for meat and meat products. Nitrate is readily reduced to nitrite (NO_2) by microorganisms present in the meat that gives the meat red color by producing NO-myoglobin (Honikel, 2008). There are regulations for the addition of $NaNO_3$ and $NaNO_2$ to meat from United States Department of Agriculture (USDA)/ Food Safety and Inspection service (FSIS). USDA/FSIS regulates $\leq$ 200 ppm for $NaNO_2$ and $\leq$ 500 ppm for $NaNO_3$ in meat products (Nyachuba et al., 2007). However, it would be better to use lower concentration of nitrate or nitrite (Cammack et al., 1999).

In recent years, Hurdle technology or combined treatments are interested for application in food preservation against foodborne pathogenic bacteria. Hurdle technology helps to prevent the bacterial resistance to individual treatments or individual antibacterial agents. Hurdle technology can also help to reduce dose or concentration of individual treatments in a combined treatment since the combined treatment may cause synergistic effects in reduction of pathogenic bacteria in food products (Zhou et al., 2010).

Further, in order to improve the activity of the antimicrobial compounds during storage, microencapsulation in edible polymers has been found one of the most effective technologies.

Microencapsulation could also decrease the organoleptically affection of antimicrobial factors, causes the slow rate release of EOs during storage time, protecting antimicrobial agents to be impaired by contacting the food matrix, promote the efficiency of them and so on (Hyldgaard et al., 2012). Hydrocolloids such as proteins, cellulose derivatives, alginates, pectins, starches, and other polysaccharides are basic components of edible coating or polymeric matrix for encapsulating antimicrobial agents (Neetoo et al., 2010). In this study alginate was used for encapsulating sixteen antimicrobial formulations containing EOs, nisin, organic acid salts and nitrite. Based on the results of this previous work, the best combination of EOs (Chinese cinnamon and Cinnamon bark) among 32 tested EOs was selected based on their additive effects against 5 pathogenic and spoilage bacteria, was selected in this work.

The objective of this study was to develop antilisterial formulations containing essential oils, nisin, nitrite and organic acid salts in meat model (fresh pork sausage) by using a standard full factorial design. Sensorial evaluation was carried out in order to verify the organoleptic acceptance of the optimized antimicrobial formulation.

MATERIAL AND METHODS

Materials

Tryptic soy broth (TSB), Peptone and Palcam agar were purchased from Alpha Biosciences Inc. (Baltimore, MD, USA). Alginate and $CaCl_2$ were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). CNC was supplied from FPInnovations pilot plant (Pointe-Claire, QC, Canada). Nisin (Niprosin™, purity 2.5%, 77.5% salt and 20% vegetable protein, Profood, Naperville, IL, USA) was purchased from Pro-food International Inc. Ground lean pork meat was purchased from a local grocery store (IGA, Laval, Quebec, Canada). Binding agent and sodium erythorbate were delivered from BSA Food Ingredients (St-Leonard, Quebec, Canada). Chinese

cinnamon and Cinnamon bark EOs were provided by Aliksir Inc. (Grondines, Québec, Canada). EOs were mixed together at a ratio of 1: 4. The chemical components of the EOs are presented in Table 1.

Bacterial strain

L. monocytogenes (HPB 2812) was stored at -80 °C in Tryptic Soy Broth (TSB) medium (TSB; BD, Franklin Lakes, NJ, USA) containing glycerol (10% v/v). Before each experiment the bacteria were propagated through two consecutive 24 h growth periods in 9 ml of TSB at 37 °C. The final concentration of bacteria after two times of propagation was approximately 10^9 CFU/ml. The culture was used as working culture for inoculation into sausage.

Experimental design for antimicrobial formulations

The preliminary experiments on using different organic acid salts as antimicrobial agents in meat, it was found that the mixture of 0.40 % (w/w) sodium acetate and 2.70 % (w/w) potassium lactate caused a bacterial reduction by less than 0.5 log CFU/g at day 7, which is better than other mixtures. This mixture was also organoleptically accepted (data not shown). Therefore, this mixture was selected for further study. Nitrite, at the concentration of 200 ppm could only decrease the growth of *L. monocytogenes* at day 7 by less than 0.5 log CFU/g meat (data not shown). In our previous results, mixed EOs of Chinese cinnamon and Cinnamon bark (0.05 %, v/w) could reduce the growth of *L. monocytogenes* by less than 0.5 log CFU/g meat during 7 days of storage at 4^oC (data not shown). In case of nisin, it was found that 1000 IU/g minced beef (25 ppm) can reduce around 1 log CFU/g minced fish during storage of 12 days (Abdollahzadeh et al., 2014). Thus, in this current study, we decided to use 25 ppm nisin, 200 ppm nitrite, 0.05 %, v/w mixed EOs, and 3.1 %, w/w mixed organic acid salts (potassium lactate plus sodium acetate) at high concentrations in the experimental design. A standard experiment

with 4 independent factors at 2 levels (4^2) was conducted using STATISCA 8 (STATSOFT Inc., Thulsa, US). The dependent factor was the count (log CFU/g) of *L. monocytogenes* at day 7. The values of the independent factors are presented in Table 2. The independent variables are nitrite (100 and 200 ppm), nisin (12.5-25 ppm), a mixture of potassium lactate and sodium acetate (1.55 and 3.1% w/v) and a mixture of EOs (0.025 and 0.05 %, v/w) in the 16 runs of the experimental design (Table 3). The low values are half of the high values.

Nisin Preparation

Nisin solution was prepared with $CaCl_2$ according to (Huq, 2014). Nisin (2% w/v) was prepared by mixing Niprosin™ powder (which contain 2.5% pure nisin) in 100mL 0.01M $CaCl_2$ solution and the pH of the nisin-$CaCl_2$ solution was adjusted to around 3 by diluted lactic acid. The nisin-$CaCl_2$ solution was centrifuged for 15 min at 3500×g at 4°C to remove the undissolved particles and collected the nisin-$CaCl_2$ supernatant.

Microencapsulation of antimicrobial formulations

All the antimicrobial factors were microencapsulated into alginate–CNC (Cellulose Nanocrystal) microbeads followed by Huq, (2014) before adding to the food model. The microbeads suspension was prepared by mixing 2 % (w/v) of alginate (guluronic acid ~ 65 – 70 %; mannuronic acid content ~5 – 35 %) in deionized water under magnetic stirring. A 1% (w/v) CNC suspension was prepared by dispersing spray dried CNC powder in deionized water under magnetic stirring. Then, the CNC suspension was subjected to ultra-sonication (QSonica Q-500, Misonix, Qsonica, LLC, Newtown, CT, USA) at 1000 J/g of CNC. A 5 % (w/w) CNC from 1 % CNC suspension (according to wt% of alginate) (Bezerra et al., 2008) and 2.5 % (w/w) of Tween 80 (emulsifier) were mixed with 2% (w/v) alginate suspension. All 16 different formulations containing nitrite, nisin, organic acid salts and mixed EOs were prepared separately. The

proportional amount of each antimicrobial formulation was added to alginate-CNC suspension and homogenized by Ultra-Turrax TP18/1059 homogenizer (Janke & Kunkel, Staufen, Germany) at 25000 rpm for 3 min. All calculations were done in terms of sausage weight (20g).

Preparation of sausage with different microencapsulated antimicrobials

The sausage was prepared by mixing lean ground pork (70% w/w), binding agent (8 % w/w) and water (22 % w/w). Sodium erythorbate (750 ppm) was mixed with sausage according to total meat weight. Then each 20g of sausage was put in a bag and kept at -80 °C under vacuumed packaging. For sterilization, the sausages were irradiated at 45 kGy before applying the antimicrobial formulation and inoculating with bacteria.

4 ml of emulsified microbeads were applied on each 20g of sausage and mixed by Lab-blender 400 Stomacher (Laboratory Equipment, London, UK) for 2 minutes at 230 rpm. Then the sausage samples were inoculated with *L. monocytogenes* to achieve a final concentration of approximately 10^3 CFU/g and mixed for another 2 min at 230 rpm. Finally, the samples were packed under vacuum and stored at 4 °C. Control sausage samples contain 4 ml of microbeads include CNC (5%) Tween 80 (2.5%), $CaCl_2$ (pH around 3) but without antibacterial agents. Since it is considered the meat model as fresh sausage in which the shelf life is normally less than 7 days (Savic, 1985). Thus, microbial analysis was conducted at day 7 of storage.

Microbiological analysis

Each sample was transferred to a stomacher bag and diluted with peptone water (0.1 % w/v). The sample was homogenized in Lab-blender 400 Stomacher for 1 min at 230 rpm. From each homogenate sample, serial decimal dilutions were done in peptone water (0.1 % w/v). Then 100 µl of each dilution was spreaded on Palcam agar plate. Palcam agar was prepared by the addition with antibiotics acriflavine (5 mg/ml), polymyxin B (10 mg/ml) and ceftazidime (8 mg/ml) in

order to get the selective enumeration of *L. monocytogenes*. After 48 h incubation at 37 °C, bacterial colonies were counted and expressed as log CFU/g of sausage.

Sensory evaluation

After optimizing the best antilisterial formulations, the sensorial analysis was performed to verify if the formulation would change the organoleptic properties of meat or not. The treated sausages were prepared in the same way as microbial analysis with the optimized 2 different antimicrobial formulations. The sausages were cooked at 400° F (~200 °C) for 10-15 minutes and 15 g of each sample was served warm to panelists. Each sample was coded with a 3 digit random number. The jury team consisted of 35 examiners who were trained for evaluating organoleptic properties of food (Département Techniques de diététique et Gestion d'un établissement de restauration, Collège Montmorency). The panelists scored the sensory odor, texture and taste of samples by using 9-point hedonic scale (9= Like extremely, 8=Like very much, 7=Like moderately, 6=Like slightly, 5=Neither like nor dislike, 4=Dislike slightly, 3=Dislike moderately, 2=Dislike very much, 1=Dislike extremely). The jury team was served unsalted biscuits and water between each sample.

Statistical analysis

The obtained data of the growth or the concentration of *L. monocytogenes* (log CFU/g) in 16 formulations of the experimental design were used for analysis of variance (ANOVA) and regression analysis using software STATISTICA 8 (STATSOFT Inc., Thulsa, US). An equation (or a model) consisted of linear effect of each independent factor and interactive effects among independent factors were built to predict the growth of *L. monocytogenes* (Equation 1).

$$Y = A_0 + \sum_{i=1}^{4} A_i \ X_i + \sum_{i=1}^{3} \sum_{j=i+1}^{4} A_{ij} X_i X_j \quad \text{(Equation 1)}$$

Where *Y*, predicted response (growth of *L. monocytogenes*, log CFU/g sausage); A_o, constant coefficient; X_i and X_j, values of various levels of the independent variables; *Ai*, values of linear coefficients; Aij, interactive coefficient between two independent factors.

For sensorial analysis, the results were expressed as mean ± SD. One-way analysis of variance (ANOVA) tests using SPSS program (IBM Corporation, Somers, NY, USA) was conducted to analyze the data of sensorial analysis results. Duncan's multiple range tests was used to compare the mean values. Differences between mean values at $P \leq 0.05$ were considered significant.

RESULTS

The results of bacterial growth at day 7 of storage for each antimicrobial formulation are presented in Table 3.

Regression analysis of the experimental design

ANOVA analysis performed on the data (growth of *L. monocytogenes*, log CFU/g) obtained at day 7 showed the regression coefficient (R^2) of the model was 0.95. The R^2 is the percent of the response variation explained by the model and represents how well the model fits with the data. Regression analysis were also carried out in order to determine the significance of the linear, and interactive coefficients of independent factors on the growth of *L. monocytogenes* and build a predictive equation (a model). Table 3 represents the regression coefficients of linear and interactive effects of 4 independent factors (nitrite, nisin, organic acid salts (OAS) and EOs) of the model. The linear effect of nitrite, nisin and OAS are not important in the model since the *P* values of these factors are higher than 0.3 whereas EOs showed the linear negative effect with $P < 0.002$. This means that EO mixtures are the most important factor in the model in which EOs could reduce the growth of *L. monocytogenes*. It was observed that there are significant interactive effects among 4 independent factors on the growth of *L. monocytogenes* in the

sausage. The interactive effects of nitrite x nisin ($P \leq 0.1$) or nisin x OAS ($P \leq 0.05$) caused a decrease in the growth of *L. monocytogenes*. The interactive effects between nitrite and EOs, or nisin and EOs or OAS and EOs are positive interactive effects at $P \leq 0.1$ (Table 3).

Generally, the regression coefficients of linear or interactive effects will be included in the equation when their *P* values are less than or equal 0.05 ($P \leq 0.05$), however, in some cases, it is necessary to consider other factors even their *P* values are smaller or equal to 0.1 ($P \leq 0.01$) to ensure a good fit equation for the prediction. In this case, it is found that all factors with *P* values ≤ 0.1 are necessary to include into the equation to predict the growth (or cell concentration) of *L. monocytogenes* in the sausage. The final model is presented in the following equation:

$$\mathbf{Y = 5.023 - 101.45X_4 - 0.02X_1X_3 + 0.168\ X_1X_4 - 0.024\ X_2X_3 + 1.33\ X_2X_4 + 12.51\ X_3X_4}$$

Where:

Y is the dependent factor of the model (the concentration of *L. monocytogenes*, log CFU/g in sausage meat product)

X_1 is the concentration of nitrite (ppm)

X_2 is the concentration of nisin (ppm)

X_3 is the concentration of OAS (%, v/w)

X_4 is the concentration of essential oil (%, v/w)

Response surface plots

As mentioned in the materials and methods section, in parallel with the experimental design, a control sausage containing encapsulation matrix (alginate-Ca) without antimicrobial agents was also conducted. The objective was to compare it with the results obtained in the experimental design in term of log CFU/g reduction when it is necessary. The concentration of bacteria in the

control sausage at day 7 was 4.3 ± 0.2 (CFU/g of meat). To see the interaction effects among independent factors on the growth of *L. monocytogenes*, response surface plots were created and presented in Figures 1 and 2.

Figure 1 presents the antibacterial effect of nisin and OAS on the growth of *L. monocytogenes* when nitrite and EOs are fixed at low concentration of 100 ppm and 0.025 %, respectively. It can be observed that at this condition, high nisin concentration (24-26 ppm) and high OAS concentration (2.8-3.2 %, w/w) could cause the growth of *L. monocytogenes* to drop to less than 1.8 log CFU/g sausage which is less than that of control sausage by more than 2.5 log CFU, or when nisin concentrations from 20 to 22 ppm and OAS concentrations from 2.4 to 3.2 % caused the growth of *L. monocytogenes* to drop to less than 2.2 log CFU/g sausage which is less than that of control sausage by more than 2 log. While these results are interesting, it should be noted that these cases required high nisin and OAS concentrations. Using nisin concentrations from 14 to 16 ppm and with OAS from low to high concentration (1.2-3.2 %), the growth of *L. monocytogenes* in sausage is less than 2.6 log CFU/g, which was less than that of control sausage by more than 1.7 log. This effect demonstrates that OAS is less important than nisin in controlling the growth of *L. monocytogenes* (Figure 1).

Figure 2 presents the antibacterial effects of nitrite and EOs on the growth of *L. monocytogenes* when nisin and OAS are fixed at low concentration of 12.5 ppm and 1.55 %, respectively. In this condition, it can be observed that when EOs concentrations are from 0.045 to 0.05 % (v/w) and with low nitrite concentrations from 100 to 160 ppm, the concentration of *L. monocytogenes* in sausage was less than 1.7 log CFU/g, which was less than that of control sausage by more than 2.6 log CFU/g. This fact demonstrated that EOs were the most important factor in reducing the growth of *L. monocytogenes* which is similar to the results obtained from regression analysis. It

is also apparent that nitrite is not important factor in reducing the growth of *L. monocytogenes* since its range can be changed from 100 to 160 ppm. Thus, the formulation containing EOs (0.05 %, v/w), low concentrations of nitrite (100 ppm), nisin (12.5 ppm) and OAS (1.55 %, w/w) can be considered as the best formulation in decreasing the growth of *L. monocytogenes* (Figure 2). This formulation (Formulation A) was then chosen for sensorial evaluation in meat products.

It is interesting to find that when EOs (0.025 %, v/w) combined with low concentrations of nitrite (100 ppm), nisin (12.5 ppm) and OAS (1.55 %, w/w) could retard the growth of *L. monocytogenes* by approximately 2.8 log CFU/g, which is still less than that of control by 1.5 log CFU/g (Figure 2). This formulation (Formulation B) was also chosen for sensorial evaluation in meat products to compare with the best formulation above.

Sensorial properties of selected antilisterial formulation in meat products

The results of sensorial analysis are presented in Table 5. The results were the average of scores based on a 9-point hedonic scale which the panelists gave to each sample. The results showed the organoleptic acceptance of formulations compared with the control. Indeed according to the scale, the values of more than 5 were considered organoleptically acceptable. Results showed both of the selected formulations were acceptable in term of texture, smell and taste in both fresh beef sausage and fresh pork sausage.

DISCUSSION

The factorial design used in this study was performed in order to optimize the concentration of 4 different antimicrobial agents to find the best antimicrobial formulation against *L. monocytogenes* in fresh pork sausage. All the formulations were able to decrease listerial growth by at least 1 log of bacteria as compare to the control sausage (4.3 log CFU/g) after 7 days of storage (Table 3). The antimicrobial formulations with lower concentration of nitrite,

nisin, organic acid salts and essential oil were effective against *L. monocytogenes* as well which demonstrated that we can reduce the acceptable concentration of each tested antimicrobial agent to half and still have antimicrobial safety (more than 1.5 log reduction as compared to the control).

Among the various methods of food preservation, Hurdle technology is one of the best methods available to prolong the shelf life. Combining various antimicrobial agents with different cell targets provides a more promising way to inhibit the growth of bacteria rather than using one component alone. In a specific example, the high efficiency of cinnamon as herbal extract against Gram positive bacteria has been demonstrated (Hernández-Ochoa et al., 2011) and indeed the suitability of them for use as preservatives in meat and meat products has been reported (Jayasena and Jo, 2013). Chinese cinnamon and Cinnamon bark at the organoleptic acceptable concentration (0.05%) was used in this study as the highest antimicrobial concentration. Combinations of different processes (Hurdle technology) can have synergistic or additive antimicrobial effects and therefore, ensure microbial safety (Jayasena and Jo, 2013). So each of the selected antimicrobial factors in this current study were used at two different concentrations to find the optimum concentration of the combination that would control the growth of *L. monocytogenes* in fresh pork sausages more efficiently and economically.

Cinnamaldehyde is the major component of selected EOs. Chinese cinnamon and Cinnamon bark contain 87.58 % and 40.71 % of trans-cinnamaldehyde respectively (Table 1). Aldehyde (CHO-) could covalently cross-link with bacterial DNA and proteins. According to the literature, cinnamaldehyde could have different effects on cell growth and metabolism at different concentrations. At low concentrations it can damage cytokinesis by inhibiting the respective enzymes. At higher concentrations it inhibits the ATPase and at lethal concentrations, it can

disrupt the cell membrane (Hyldgaard et al., 2012). At high but sub-lethal concentrations, cinnamaldehyde can enter the preplasmic space and decrease the activity of transmembrane ATPase. It is hard to say if the inhibition of ATPase is the main cause of cell death as the higher concentration of cinnamaldehyde can also cause membrane permeabilisation (Hyldgaard et al., 2012).

According to Burt (2004) and Hyldgaard et al. (2012), EOs control bacterial growth by damaging the cell membrane, inhibit some of the enzymes such as histidine decarboxylase, ATPase and cell wall synthesizing enzymes, and produce covalent cross-links with DNA. Increasing the permeability and depolarization of cell membranes are two main ways by which antimicrobial compounds act on the membrane (Hyldgaard et al., 2012). Certain components of EOs could also have an effect on the transport of nutrients and ions and in general permeabilize the cell membrane (Hyldgaard et al., 2012).

When comparing food systems (meat model) to *in vitro* conditions, greater concentrations of EOs are needed to obtain the same inhibitory effect against target bacteria (de Oliveira et al., 2011; Jayasena and Jo, 2013). In addition, the presence of fat, starch and protein in food products can impair the EO's components (Hyldgaard et al., 2012). Indeed certain foods may contain more nutritive components than that found in laboratory culture media, which could help the bacteria attain their maximum replication rate and repair the cell damage (Gill et al., 2002). Some studies showed the efficiency of EOs against *L. monocytogenes* can be reduced in certain food products with high levels of fat (de Oliveira et al., 2011; Jayasena and Jo, 2013). This could be due to the fact that EOs are more soluble in the lipid phase of foods, whereas bacteria are more localized to the aqueous phase (de Oliveira et al., 2011). The activity of starch on EOs is not pronounced as that found with lipids but, starch especially at high concentration can also

reduce antilisterial activity of EOs by protecting the bacteria (de Oliveira et al., 2011; Devlieghere et al., 2004; Gutierrez et al., 2008). According to Gutierrez et al. (2008) the presence of protein can enhance the growth of *L. monocytogenes* while the existence of them in culture media improves the activity of EOs as some peptides have hydrophobic properties and can help in the dissolution of EOs. Therefore in order to maximize food safety without increasing the concentration of EOs over the organoleptically acceptable limit, combining other antimicrobial agents with EOs is necessary.

Encapsulating EO or other antimicrobial components like nisin protects them from the effects of components of the food matrix such as fat, starch and protein (Hyldgaard et al., 2012). Edible polymers were used in this study as it has been demonstrated that encapsulated antimicrobial agents have a higher inhibitory activity than non-encapsulated antimicrobial agents (Huq, 2014). Indeed, entrapping EOs and other antimicrobial compounds in edible polymer reduce the probable negative organoleptic effect of each of them on food. Furthermore entrapping the EOs in edible polymer causes a slow rate of release encapsulated EOs, thus prolonging their activity during long refrigerated storage time while antimicrobial dips or sprays cannot be used for long time periods as the diffusion would continue into the food enabling microbial growth on the surface (Neetoo et al., 2010). Alginate was used in this study as it has no detectable taste and due to probable interaction between incorporated antimicrobials and alginate, it is an efficient carrier for various antimicrobial agents (Neetoo et al., 2010). For instance, alginate-entrapped nisin has been use to treat poultry meat with antimicrobial compounds (Huq et al., 2012).

Bacteriocins are acceptable as natural food biopreservatives (García et al., 2010). Nisin causes cell death by binding to the peptidoglycan layer and causing destabilization of the cytoplasmic membrane by forming pores (Solomakos et al., 2008a), resulting in leakage of intercellular

metabolites causesing cell death. It has been shown that nisin has antilisterial activity in a meat system (Solomakos et al., 2008b). The effect of nisin could be weakened due to its binding with proteins, fat and reaction with meat proteases (Solomakos et al., 2008b). The efficiency of nisin can be affected by pH, food processing and food ingredients too (Abdollahzadeh et al., 2014). So as it was previously mentioned, encapsulating nisin in edible polymer could protect this antimicrobial from food ingredients. The effect of nisin on bacteria is also dependent on the rigidity of their membrane. Decreasing the temperature could have a negative effect on nisin activity as low temperatures might result in a decrease of the membrane fluidity (Solomakos et al., 2008b). In the case of nisin alone it would be probable to get less nisin activity *in situ*, compare to *in vitro* where the growth medium is incubated at 37 °C since sausage may contain proteases which could inhibit the activity of nisin. If nisin was combined with other antimicrobial agents like EOs, an additive effect could be observed as EOs could disintegrate the protective outer membrane which would make the bacteria more sensitive to nisin (Solomakos et al., 2008a). Inhibition of cell wall synthesis and forming the pores are two of the modes of action for nisin (García et al., 2010).

Bacteriocins alone cannot ensure the adequate safety, they have to be combined with other technologies or other antimicrobial agents such as sodium acetate or potassium lactate (Zacharof and Lovitt, 2012). Apostolidis et al. (2008) showed an increase of antimicrobial activity of combined EOs with salts of organic acids such as potassium lactate against *L. monocytogenes*. Organic acids and their salts are used as preservatives in foods to increase the lag phase of microbial proliferation. In fact lactates can inhibit the growth of bacteria by reducing the water activity of food products leading to retarded development of bacteria and also by acidifying the intracellular pH (Stekelenburg, 2003). It has been found that the probable site of action for

lactate radical (sodium lactate) is proline dehydrogenase (Apostolidis et al., 2008). Potassium lactate is derived from lactic acid that naturally present in animal tissue. It extends the lag phase of pathogenic bacteria thereby prolong the shelf life of food (Stekelenburg, 2003).

Sodium acetate is effective in inhibiting the microbial growth and extending shelf life. It has been approved by the US-FDA as a flavouring and pH control agent. The permissive level for sodium acetate based on US-FDA is 0.25% (Grosulescu et al., 2011). Several studies demonstrated the antimicrobial activity of sodium acetate in different food systems. For instance, Manju et al. (2007) used 2% of sodium acetate and combined it with vacuum-packaging to extends the shelf life of seafood to 15 days. Blom et al. (1997) demonstrated that sodium acetate individually at the concentration of 0.5% could inhibit the growth of *L. monocytogenes*. Combination of EOs with salts of organic acids provides benefits for both food safety and human health. The combination can be used as a natural multiple-barrier food preservatives (Apostolidis et al., 2008).

$NaNO_2$ or KNO_2 are perfect agents for curing (Honikel, 2008). "Curing" is an expression which used for manufacturing meat products with nitrite or nitrate (Honikel, 2008). Nitrite is a very reactive substance and produces several reactions in meat which is why its concentration should be controlled. The sum of both nitrite and nitrate is critical for human body because nitrate can be reduced to nitrite in the oral cavity and in the stomach, due to acidic environment and nitrite can form carcinogenic nitrosamines (Honikel, 2008). It should be noted that the compounds derived from nitrite during storage time are bactericidal compounds not the nitrite itself (Cammack et al., 1999). Nitrite is more toxic than nitrate (10 times) (Honikel, 2008). The lethal doses are 80-800 nitrate/kg body weight and 33-250 nitrite/kg body weight. According to Honikel (2008), the antibacterial mechanism of nitrite is not understood yet, while the scientific

literature has revealed that nitrite slows or controls the growth of *L. monocytogenes* but does not totally stop the growth of this bacterium (Myers et al., 2013).

The antibotulinal activity of nitrite in cooked meat medium is less than in bacterial growth laboratory-compounded medium due to the interaction of nitrite with the components of meat (Cui et al., 2010). It has been proven that the combination of $NaNO_2$ and EOs have greater antimicrobial activities compared to each of them individually (Cui et al., 2010).

The synergetic effect of Na and EOs is important for food companies as it causes them to be used at lower concentrations. Lower nitrite is preferable for consumers and lower EO is promising for not influencing (affecting) the sensorial properties of food products, especially meats (Cui et al., 2010).

Different antimicrobial activities of EOs plus sodium nitrite was observed in culture media and ground pork and it was found that the antimicrobial effects of this combination in ground pork was less effective as compared to medium; which could be due to nutrients in food that promote cellular repair and cause less sensitivity of tested bacteria (Cui et al., 2010). Also it has been shown that sodium erythorbate can improve the activity of nitrite at lower concentration (Redondo-Solano et al., 2013).

CONCLUSION

To promote the safety of meat products, a combination of mild preservation technologies is important. Our results demonstrated that the combination of different antimicrobial agents and encapsulation in alginate microbeads could reduce the growth of *L. monocytogenes* in fresh pork sausages significantly as compared to that of control. The formulation A (mixed EOs (0.05 %, v/w), mixed organic acid salts (1.55%, w/w), nisin (12.5 ppm) and nitrite (100 ppm)) and

formulation B (mixed EOs (0.025 %, v/w), mixed organic acid salts (1.55%, w/w), nisin (12.5 ppm) and nitrite (100 ppm)) resulted in the reduction of *L. monocytogenes* by more than 2.6 and 1.5 log CFU/g sausage as compared to that of control. The two formulations were also organoleptically accepted in both pork and beef sausages. This study showed that combination treatments could reduce the concentrations of individual antimicrobial components while still maintaining high antibacterial effects.

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References

1. Abdollahzadeh, E., Rezaei, M., Hosseini, H., 2014. Antibacterial activity of plant essential oils and extracts: The role of thyme essential oil, nisin, and their combination to control Listeria monocytogene inoculated in minced fish meat. Food Control 35, 177-183.
2. Apostolidis, E., Kwon, Y.I., Shetty, K., 2008. Inhibition of Listeria monocytogenes by oregano, cranberry and sodium lactate combination in broth and cooked ground beef systems and likely mode of action through proline metabolism. International Journal of Food Microbiology 128, 317-324.

3. Bezerra, M.A., Santelli, R.E., Oliveira, E.P., Villar, L.S., Escaleira, L.A., 2008. Response surface methodology (RSM) as a tool for optimization in analytical chemistry. Talanta 76, 965-977.
4. Blom, H., Nerbrink, E., Dainty, R., Hagtvedt, T., Borch, E., Nissen, H., Nesbakken, T., 1997. Addition of 2.5% lactate and 0.25% acetate controls growth of Listeria monocytogenes in vacuum-packed, sensory-acceptable servelat sausage and cooked ham stored at 4°C. International Journal of Food Microbiology 38, 71-76.
5. Burt, S., 2004. Essential oils: their antibacterial properties and potential applications in foods—a review. International Journal of Food Microbiology 94, 223-253.
6. Cammack, R., Joannou, C.L., Cui, X.-Y., Torres Martinez, C., Maraj, S.R., Hughes, M.N., 1999. Nitrite and nitrosyl compounds in food preservation. Biochimica et Biophysica Acta (BBA) - Bioenergetics 1411, 475-488.
7. Cui, H., Gabriel, A.A., Nakano, H., 2010. Antimicrobial efficacies of plant extracts and sodium nitrite against Clostridium botulinum. Food Control 21, 1030-1036.
8. Dave, D., Ghaly, A.E., 2011. Meat spoilage mechanisms and preservation techniques: A critical review. American Journal of Agricultural and Biological Sciences 6, 486.
9. de Oliveira, T.L.C., de Araújo Soares, R., Ramos, E.M., das Graças Cardoso, M., Alves, E., Piccoli, R.H., 2011. Antimicrobial activity of Satureja montana L. essential oil against Clostridium perfringens type A inoculated in mortadella-type sausages formulated with different levels of sodium nitrite. International Journal of Food Microbiology 144, 546-555.

10. Devlieghere, F., Vermeulen, A., Debevere, J., 2004. Chitosan: antimicrobial activity, interactions with food components and applicability as a coating on fruit and vegetables. Food Microbiology 21, 703-714.
11. Fratianni, F., De Martino, L., Melone, A., De Feo, V., Coppola, R., Nazzaro, F., 2010. Preservation of chicken breast meat treated with thyme and balm essential oils. Journal of Food Science 75, M528-535.
12. García, P., Rodríguez, L., Rodríguez, A., Martínez, B., 2010. Food biopreservation: promising strategies using bacteriocins, bacteriophages and endolysins. Trends in Food Science & Technology 21, 373-382.
13. Gill, A.O., Delaquis, P., Russo, P., Holley, R.A., 2002. Evaluation of antilisterial action of cilantro oil on vacuum packed ham. International Journal of Food Microbiology 73, 83-92.
14. Grosulescu, C., Juneja, V.K., Ravishankar, S., 2011. Effects and interactions of sodium lactate, sodium diacetate, and pediocin on the thermal inactivation of starved Listeria monocytogenes on bologna. Food Microbiology 28, 440-446.
15. Gutierrez, J., Barry-Ryan, C., Bourke, P., 2008. The antimicrobial efficacy of plant essential oil combinations and interactions with food ingredients. International Journal of Food Microbiology 124, 91-97.
16. Hernández-Ochoa, L., Aguirre-Prieto, Y.B., Nevárez-Moorillón, G.V., Gutierrez-Mendez, N., Salas-Muñoz, E., 2011. Use of essential oils and extracts from spices in meat protection. Journal of Food Science and Technology, 1-7.
17. Honikel, K.-O., 2008. The use and control of nitrate and nitrite for the processing of meat products. Meat Science 78, 68-76.

18. Huq, T., Vu, K.D., Riedl, B., Bouchard, J., Lacroix, M. (2014) Synergistic Effect of Gamma (γ)-Irradiation and Microencapsulated Antimicrobials against Listeria monocytogenes on Ready-to-Eat (RTE) Meat. Food Microbiology (accepted manuscript).
19. Huq, T., Salmieri, S., Khan, A., Khan, R.A., Le Tien, C., Riedl, B., Fraschini, C., Bouchard, J., Uribe-Calderon, J., Kamal, M.R., Lacroix, M., 2012. Nanocrystalline cellulose (NCC) reinforced alginate based biodegradable nanocomposite film. Carbohydr Polym 90, 1757-1763.
20. Hyldgaard, M., Mygind, T., Meyer, R.L., 2012. Essential oils in food preservation: mode of action, synergies, and interactions with food matrix components. Frontiers in Microbiology 3.
21. Jayasena, D.D., Jo, C., 2013. Essential oils as potential antimicrobial agents in meat and meat products: A review. Trends in Food Science & Technology 34, 96-108.
22. Manju, S., Jose, L., Srinivasa Gopal, T.K., Ravishankar, C.N., Lalitha, K.V., 2007. Effects of sodium acetate dip treatment and vacuum-packaging on chemical, microbiological, textural and sensory changes of Pearlspot (Etroplus suratensis) during chill storage. Food Chemistry 102, 27-35.
23. Millette, M., Le Tien, C., Smoragiewicz, W., Lacroix, M., 2007. Inhibition of Staphylococcus aureus on beef by nisin-containing modified alginate films and beads. Food Control 18, 878-884.
24. Myers, K., Montoya, D., Cannon, J., Dickson, J., Sebranek, J., 2013. The effect of high hydrostatic pressure, sodium nitrite and salt concentration on the growth of Listeria monocytogenes on RTE ham and turkey. Meat Science 93, 263-268.

25. Neetoo, H., Ye, M., Chen, H., 2010. Bioactive alginate coatings to control Listeria monocytogenes on cold-smoked salmon slices and fillets. International Journal of Food Microbiology 136, 326-331.
26. Nesbitt, A., Thomas, M.K., Marshall, B., Snedeker, K., Meleta, K., Watson, B., Bienefeld, M., 2014. Baseline for consumer food safety knowledge and behaviour in Canada. Food Control 38, 157-173.
27. Nyachuba, D., Donnelly, C., Howard, A., 2007. Impact of Nitrite on Detection of Listeria monocytogenes in Selected Ready-to-Eat (RTE) Meat and Seafood Products. Journal of Food Science 72, M267-M275.
28. Oussalah, M., Caillet, S., Saucier, L., Lacroix, M., 2007. Inhibitory effects of selected plant essential oils on the growth of four pathogenic bacteria: *E. coli* O157:H7, *Salmonella* Typhimurium, *Staphylococcus aureus* and *Listeria monocytogenes*. Food Control 18, 414-420.
29. Perumalla, A., Hettiarachchy, N.S., Over, K.F., Ricke, S.C., Gbur, E.E., Zhang, J., Davis, B., 2012. Effect of Potassium Lactate and Sodium Diacetate Combination to Inhibit Listeria Monocytogenes In Low and High Fat Chicken and Turkey Hotdog Model Systems. Open Food Science Journal 6, 16-23.
30. Ramaswamy, V., Cresence, V.M., Rejitha, J.S., Lekshmi, M.U., Dharsana, K.S., Prasad, S.P., Vijila, H.M., 2007. Listeria--review of epidemiology and pathogenesis. Journal of Microbiology, Immunology and Infection 40, 4-13.
31. Redondo-Solano, M., Valenzuela-Martinez, C., Cassada, D.A., Snow, D.D., Juneja, V.K., Burson, D.E., Thippareddi, H., 2013. Effect of meat ingredients (sodium nitrite and erythorbate) and processing (vacuum storage and packaging atmosphere) on germination

and outgrowth of Clostridium perfringens spores in ham during abusive cooling. Food Microbiology 35, 108-115.

32. Samaxa, R.G., Matsheka, M.I., Mpoloka, S.W., Gashe, B.A., 2012. Prevalence and Antimicrobial Susceptibility of Salmonella isolated from a variety of raw meat sausages in Gaborone (Botswana) retail stores. Journal of Food Protection 75, 637-642.
33. Savic, I.V., 1985. Small-scale sausage production. Food and Agriculture Organization (FAO) of the United Nation.
34. Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R.V., Widdowson, M.-A., Roy, S.L., Jones, J.L., Griffin, P.M., 2011. Foodborne illness acquired in the United States—major pathogens. Emerg Infect Dis 17.
35. Solomakos, N., Govaris, A., Koidis, P., Botsoglou, N., 2008a. The antimicrobial effect of thyme essential oil, nisin and their combination against Escherichia coli O157:H7 in minced beef during refrigerated storage. Meat Sci 80, 159-166.
36. Solomakos, N., Govaris, A., Koidis, P., Botsoglou, N., 2008b. The antimicrobial effect of thyme essential oil, nisin, and their combination against Listeria monocytogenes in minced beef during refrigerated storage. Food Microbiology 25, 120-127.
37. Stekelenburg, F.K., 2003. Enhanced inhibition of Listeria monocytogenes in Frankfurter sausage by the addition of potassium lactate and sodium diacetate mixtures. Food Microbiology 20, 133-137.
38. Zacharof, M., Lovitt, R., 2012. Bacteriocins produced by lactic acid bacteria a Review Article. APCBEE Procedia 2, 50-56.
39. Zhou, G.H., Xu, X.L., Liu, Y., 2010. Preservation technologies for fresh meat – A review. Meat Science 86, 119-128.

Table 1. Essential oils and their composition

Latin name	**Common name**	**Origin**	**Distilled part**	**Composition (%)**[1]
Cinnamomum cassia	Chinese cinnamon	Vietnam	Bark	*Trans*-cinnamaldehyde (87.58), cinnamyl acetate (7.53)
Cinnamomum verum	Cinnamon bark	Madagascar	Bark	*Trans*-cinnamaldehyde (40.71), cinnamyl acetate (14.25), β-phellandrene (9.02), β-caryophyllene (7.41)

[1]Composition determined by gas chromatography analysis using 2 capillary columns (30 m × 0.25 mm): Supelcowax 10 (polar) and DB-5 (apolar).

Table 2: Antimicrobial agents and their values

Independent factors	**-1 (low value)**	**+1 (high value)**
Nitrite (ppm)	100	200
Nisin (ppm)	12.5	25
Mixed potassium lactate and sodium acetate (%, w/w)	1.55	3.1
Mixed essential oils (%, v/w)	0.025	0.05

Table 3. Final concentration of *Listeria monocytogenes* (log CFU/g meat) in sausage samples at day 7 stored at 4 °C

Formulations	Nitrite (ppm)	Nisin (ppm)	Organic acid salts (%, w/w)	Essential oil (%, v/w)	log CFU/g meat
1	100	12.5	1.55	0.025	2.6 ± 0.2
2	200	12.5	1.55	0.025	3.1 ± 0.1
3	100	25	1.55	0.025	2.6 ± 0.2
4	200	25	1.55	0.025	2.4 ± 0.1
5	100	12.5	3.1	0.025	2.9 ± 0.3
6	200	12.5	3.1	0.025	2.4 ± 0.3
7	100	25	3.1	0.025	1.8 ± 0.3
8	200	25	3.1	0.025	1.5 ± 0.0
9	100	12.5	1.55	0.05	1.8 ± 0.2
10	200	12.5	1.55	0.05	2.1 ± 0.3
11	100	25	1.55	0.05	1.6 ± 0.2
12	200	25	1.55	0.05	2.1 ± 0.3
13	100	12.5	3.1	0.05	1.9 ± 0.3
14	200	12.5	3.1	0.05	2.2 ± 0.3
15	100	25	3.1	0.05	1.6 ± 0.2
16	200	25	3.1	0.05	1.7 ± 0.2

Table 4. Regression coefficient of linear and interactive effects of 4 independent factors of the equation

Factors	**Regression coefficient**	***P* value**
Intercept	5.02	0.0018
Linear effects		
Nitrite (X_1)	0.00	0.9733
Nisin (X_2)	-0.03	0.3899
Organic acid salts (OAS) (X_3)	0.16	0.6215
Essential Oil (X_4)	-101.46	0.0017
Interactive effects		
X_1X_3	-0.002	0.0886
X_1X_4	0.168	0.0581
X_2X_3	-0.02	0.0399
X_2X_4	1.33	0.0604

Table 5. Sensorial evaluation of two kinds of fresh sausage with two of the selected antimicrobial formulations

	Texture		**Smell**		**Taste**	
	Pork	**Beef**	**Pork**	**Beef**	**Pork**	**Beef**
FA[1]	6.08 ± 1.88[a]	6.22 ± 1.64[a]	6.50 ± 1.88[a]	5.69 ± 1.80[a]	5.83 ± 2.43[a]	4.86 ± 2.03[a]
FB	6.44 ± 2.02[a2]	6.41 ± 1.40[a]	6.61 ± 1.51[a]	5.58 ± 1.66[a]	5.86 ± 2.11[a]	5.19 ± 2.26[a]
FC	6.25 ± 1.55[a]	6.44 ± 1.59[a]	5.91 ± 1.99[a]	5.91 ± 1.96[a]	5.16 ± 1.91[a]	5.05 ± 2.30[a]

[1]FA is the formulation A, containing low concentrations nitrite (100 ppm), nisin (12.5 ppm), organic acid salts (1.55%), and high concentration of EOs (0.05 %, v/w). FB is the formulation B, containing low concentrations of EOs (0.025 %, v/w), nitrite (100 ppm), nisin (12.5 ppm) and organic acids salt (1.55%). FC is the control without antimicrobial agents.

[2]In the same column bearing the same lower case letters are not significantly different ($p > 0.05$).

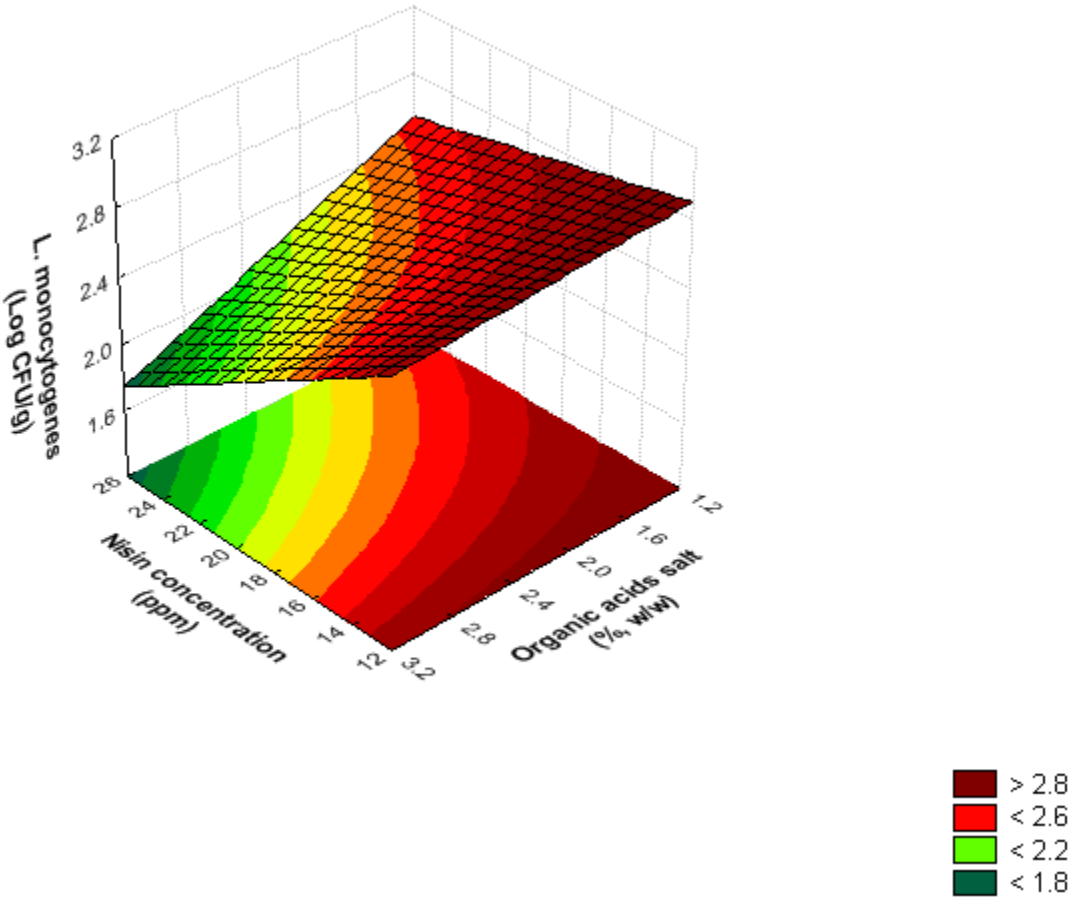

Figure 1. Effect of nisin and organic acids salt on the growth of *L. monocytogenes* (log CFU/g meat) (nitrite and EOs are fixed at low levels, 100 ppm and 0.025 %, respectively)

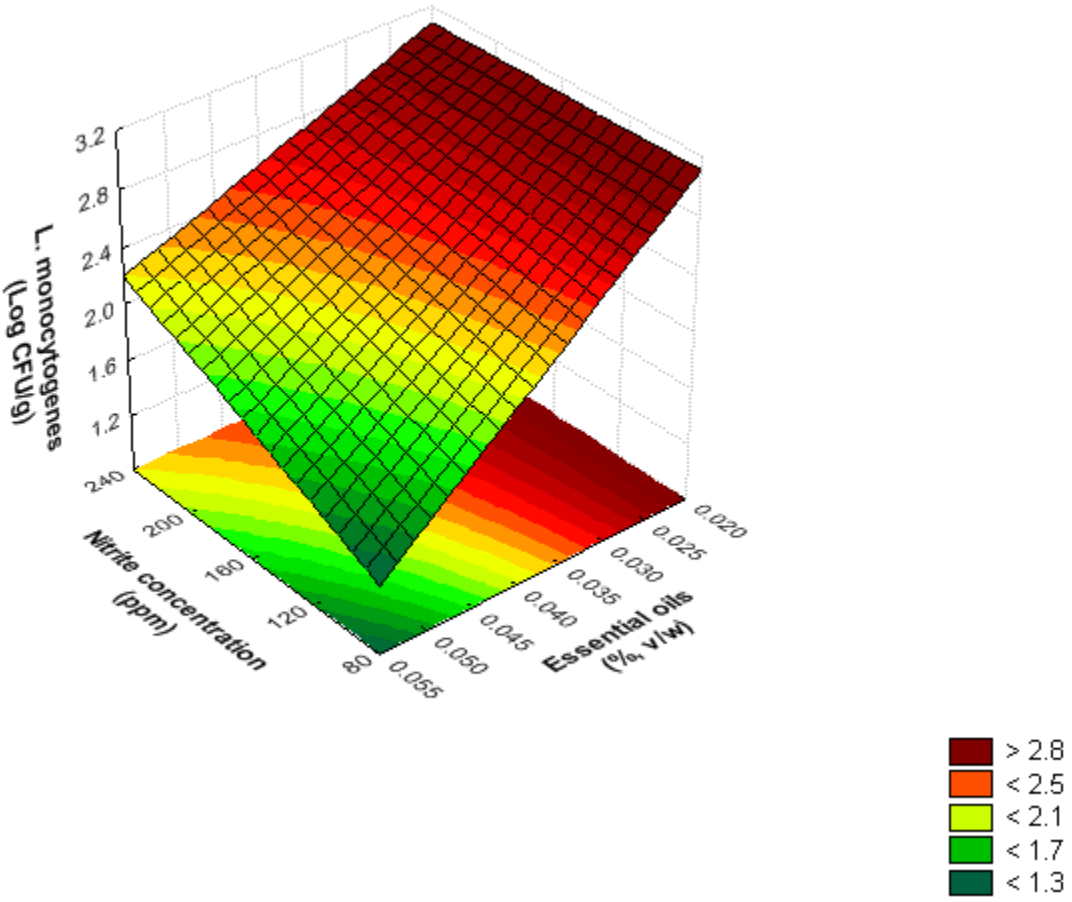

Figure 2. Effect of nitrite and EOs on the growth of *L. monocytogenes* (log CFU/g meat) (nisin and organic acid salt are fixed at low levels, 12.5 ppm and 1.54 %, respectively)

CHAPTER 4: DISCUSSION

In this study the antimicrobial activity of 32 EOs were first evaluated *in vitro* against 5 pathogenic and spoilage bacteria (*Listeria monocytogenes, Staphylococcus aureus, Escherichia coli, Salmonella* Typhimurium and *Pseudomonas aeruginosa)* following 4 different methods (agar diffusion assay, micro-atmosphere assay, broth microdilution assay and checkerboard). Furthermore, in order to find the best antimicrobial formulation, the selected combination of EOs was evaluated *in situ* (fresh pork sausage) in combination with 3 other antimicrobial agents (nitrite, nisin and organic acid salts).

As it was mentioned in the first article, some EOs such as Red thyme, Red bergamot, Winter savory, Chinese cinnamon and Cinnamon bark were more effective than the others. Since EOs are consist of different components, it is difficult to find the specific target for them. Also most of their constituents have several targets (Hyldgaard et al., 2012). As EOs have different cell targets, no particular resistance has been reported yet which makes them one of the more promising candidates for the preservation of food (Bakkali et al., 2008). In general, the antimicrobial activity of EOs is linked to their lipophilic characteristic. EOs accumulate in the cell membrane and permeabilize it. It has been showed that contacting bacteria with EOs leads to the release of cell constituents. Bakkali et al. (2008) and Turgis et al. (2009) showed the release of cell constituents by measuring the intracellular pH and ATP concentration levels of bacteria after treatment with EOs. Similar effects of EOs on bacteria were found by analysis of cell membrane morphology and lipid content (Bakkali et al., 2008; Turgis et al., 2009).

According to all results obtained for *in vitro* tests EOs were generally more effective against Gram-positive bacteria (*L. monocytogenes* and *S. aureus*) rather than on Gram-negative bacteria (*E. coli*, *S.* Typhimurium and *P. aeruginosa*). However, among Gram-negatives, *E. coli* showed

more sensitivity to tested EOs. The inhibition mechanism of EOs is different in Gram-positives compared to Gram-negatives. EOs have mostly hydrophobic constituents and they accumulate in the Gram-positive membrane, affecting its integrity and they also disrupt the membranes of bacterial cells. In Gram-negative bacteria, besides affecting membrane integrity, EOs enter the bacteria through the porin proteins and bind to cellular metabolic enzymes, affecting their activity (Lacroix, 2007; Oussalah et al., 2006). Specifically, EOs contain many different classes of chemical compounds and their major components are thought to be responsible for their antimicrobial activity.

Within all the 32 tested EOs, there were some EOs which demonstrated higher efficiency than the others against the most bacteria tested in this study. Red thyme, Red bergamot, Winter savory, Chinese cinnamon and Cinnamon bark which their major component was thymol 48.03%, carvacrol 48.21%, carvacrol 26.8%, *Trans*-cinnamaldehyde 87.58% and *Trans*-cinnamaldehyde 40.71% respectively were mostly efficient in controlling the growth of bacteria.

According to Hyldgaard et al. (2012), the active component of EOs can be classified into four groups based on their chemical structure: terpenes, terpenoids, phenylpropenes, and others. Among EOs evaluated, some of them contain high quantities of hydrocarbons such as Melissa (β-caryophyllene 23.31%), Common juniper (α-pinene 75.61%), Balsam fir (β-pinene 31.41%), Red pine (α-pinene 49.49%), White pine (α-pinene 29.82%), and Ajowan (γ-terpinene 36.40%). With the exception of Ajowan which showed high efficiency against tested bacteria in agar diffusion and micro-atmosphere method, the other EOs used in this study did not display any significant antimicrobial activity in any of the methods used. Generally, EOs with high content of hydrocarbons demonstrate weak antimicrobial activity compared to the other EOs containing high concentration of terpenoids (Bassolé and Juliani, 2012). Terpenes are hydrocarbons and are

made from 5-carbon-base units (isoprene) (Bakkali et al., 2008). Terpenes such as α-pinene, β-pinene, p-cymene, γ-terpinene, and β-caryophyllene don't display high antimicrobial activity. Using them alone is not an efficient way to inhibit the growth of bacteria (Hyldgaard et al., 2012). It should be mentioned that these compounds could exist in other EOs at lower concentration (minor component) too.

Another active compound of EOs is terpenoid which is a terpene containing oxygen and can be subdivided to alcohols, phenols, aldehydes, ethers, esters, ketones. Carvacrol, thymol, geraniol, and menthol are some of the examples for terpenoids. Terpenoids are active against a broad spectrum of microorganisms (Hyldgaard et al., 2012). In our study, Winter savory, Red bergamot and Oregano EOs contain 26.8%, 48.21%, and 21.01% of carvacrol respectively as their major components. Winter savory and Red bergamot demonstrated high efficiency against tested bacteria either Gram-positives or Gram-negatives with the exception of *P. aeruginosa* which was the most resistant to EOs among our tested bacteria, while Oregano was mostly effective against *S. aureus*, *E. coli* and *S.* Typhimurium. Carvacrol is an isoprenyl phenol which shows an antimicrobial activity that is more effective under acidic conditions has antimicrobial activity. Carvacrol is more efficient under acidic condition (pH 4.0). Cell membranes are the major target of carvacrol as it disintegrates the outer membrane by modifying the morphology of the cell membrane. Carvacrol is hydrophobic and could permeabilize and depolarize the cell membrane. Carvacrol permeabilizes the membrane to protons and potassium. It may reduce ATP synthesis so the amount of ATP will decrease in the cell (Oussalah et al., 2006). It could also interact with membrane proteins and enzymes (Hyldgaard et al., 2012). Its activity is dependent on its concentration and time of contact. In fact the free hydroxyl group and proton exchange are the main reason for the bactericidal activity of carvacrol (Ait-Ouazzou et al., 2013). Gram-positive

bacteria like *L. monocytogenes* are more sensitive to carvacrol than Gram-negative like *E. coli*. In general, Gram-negative bacteria are more resistant to antimicrobial agents in compare to Gram-positive bacteria due to having lipopolysaccharide as a major component in their outer membrane as this constituent avoids the accumulation of the EOs on the membrane (Oussalah et al., 2007).

Due to their hydroxyl group, phenolic compounds such as carvacrol and thymol play an important role in bacterial inhibition (Jayasena and Jo, 2013). The presence and location of hydroxyl group in EOs determine their antimicrobial efficiency of EOs (Lacroix, 2007). Hydroxyl groups can deactivate enzymes and might cause cell components loss and cause change on fatty acids and phospholipids making the membrane more permeable. Also it could prevent genetic materials synthesis (Hernández-Ochoa et al., 2011; Zhang et al., 2009). In addition our results showed that Common thyme, Red thyme, and Ajowan EOs containing 34.70%, 48.03% and 32.35 % of thymol respectively, could inhibit the tested bacteria efficiency except for Common thyme when tested using broth micro-dilution method. In fact, the structure of thymol is similar to carvacrol as it has hydroxyl group on the phenolic ring. It is believed that thymol interacts with proteins in cell membrane and permeabilize the membrane, resulting in the loss of ATP and potassium ions (Hyldgaard et al., 2012). Morphological damages in the outer membrane of *L. monocytogenes* and *E. coli* in presence of thymol and carvacrol were observed by scanning electron microscopy (Oussalah et al., 2006).

Wild bergamot and Palmarosa EOs containing high concentration of geraniol as their major component, 91.71% and 80.14% respectively, demonstrated fine antimicrobial activity against the bacteria. Both could effectively inhibit the tested bacteria under broth micro-dilution and micro-atmosphere methods while they didn’t work efficiently against the tested bacteria using

the agar diffusion method. In addition, the combination of Wild bergamot with other EOs such as Red bergamot, Chinese cinnamon and Cinnamon bark showed additive effects against *S. aureus* and were able to work together efficiently. Geraniol is another example of terpenoids. It is one of the compounds which modulate drug resistance in several Gram-negative bacteria (Solorzano-Santos and Miranda-Novales, 2012).

Apart from phenolic compounds, the non phenolic compounds have also antimicrobial effects. Our study demonstrated that clove containing 83-95 % of eugenol showed high antimicrobial activity in all methods used. Also, according to the results of our checkerboard analysis, the combination of clove with Red bergamot demonstrated additive effects against *L. monocytogenes*, *E. coli* and *S.* Typhimurium.

Eugenol can inhibit the production of enzymes. It also can prevent their activity (ie. amylase and protease). This is probably due to the presence of the hydroxyl group in eugenol and its ability to bind to the proteins (Lacroix, 2007). It has been shown that eugenol can inhibit the activity of ATPase which ultimately leads to cell death, as the generation of energy is absolutely requires for cell growth and survival (Hyldgaard et al., 2012). Tajkarimi et al. (2010) showed that the non phenolic compounds that were extracted from some EOs such as Oregano, Clove, and Cinnamon possessed antimicrobial efficacy against Gram-positive and Gram-negative bacteria. In fact the third type of active component of EOs is Phenylpropanoids which are sub-family of phenylpropenes that are synthesized from the amino acid phenylalanine in plants. Phenylpropenes are present in low concentrations in EOs. Eugenol, cinnamaldehyde, vanillin are some of the phenylpropenes (Hyldgaard et al., 2012). Their activity depends on the type and on the number of substituents on the aromatic ring, type of bacteria, media, and temperature. (Hyldgaard et al., 2012)

In addition, Chinese cinnamon and Cinnamon bark EOs used in our study contain high concentration of *Trans*-cinnamaldehyde as the major component (87.58% and 40.71% respectively) and these EOs were shown to be the most effective among the most effective EOs in all methods tested *in vitro*. The combination of these two oils was also assessed and the results showed on additive effect against all tested bacteria using the checkerboard method. This combination could inhibit the growth of all tested bacteria from 0.16 (*L. monocytogenes*) to 0.52 (*E. coli*) log *in situ* (ground pork) after 7 days of storage as well.

Oussalah et al. (2006) measured the intracellular ATP of *E. coli* O157:H7 and *L. monocytogenes* before and after treatment with 0.025% (v/v) of Chinese cinnamon EO. That study showed a significant decrease in intracellular ATP concentration of *E. coli* from 1.84ng/ml to 1.09ng/ml and intracellular ATP concentration of *L. monocytogenes* from 6.24ng/ml to 4.09ng/ml. It also demonstrated that treatment with Chinese cinnamon creates permeability in the cell membrane. Hyldgaard et al. (2012) showed that in case of *E. coli* and *S.* Typhimurium cinnamaldehyde was as efficient as thymol and carvacrol. Aldehyde groups have been shown to cause covalent cross-links with DNA and proteins via their amine groups (Lacroix, 2007). Cinnamaldehyde, at low concentrations could inhibit some enzymes like those involved in cytokinesis. At higher concentrations, it can penetrate into the periplasm and can inhibit ATPase activity. Inhibition of ATPase and disruption of membrane can cause cell death (Hyldgaard et al., 2012). Biofilm formation is used by bacteria as an one way to become resistant to antimicrobials. Cinnamon oil is one of the EOs which has effects against both planktonic and biofilm culture of bacteria such as *S.epidermidis* (Solorzano-Santos and Miranda-Novales, 2012). Cinnamaldehyde can cause the cell death by increasing the content of saturated fatty acids in the cell membrane and make the

membrane more rigid in *E. coli* or disintegrating the cell membrane in *S. aureus* (Dussault et al., 2009; Hyldgaard et al., 2012).

Among all the tested bacteria evaluated in our study *P. aeruginosa* was the most resistant. In the broth micro-dilution method, just 3 EOs were able to inhibit the growth of this bacterium at the concentration of 10000 ppm (1%) or lower. In the agar diffusion assay, Red thyme, Red bergamot, Ajowan, Winter savory, Chinese cinnamon and Cinnamon bark could control the growth of *P. aeruginosa* but except Ajowan, other EOs showed a low activity. Furthermore, for the micro-atmosphere method, just the Chinese cinnamon and Red bergamot could inhibit the growth of this bacterium. Our results showed that the high antimicrobial efficiency when Chinese Cinnamon and Cinnamon bark were combined in controlling the growth of *P. aeruginosa in vitro* tests. The combination of these two EOs showed an additive effect toward this bacterium. *In situ* test results showed a bacterial reduction of 0.62, 0.33, and 0.23 log after 1, 4, and 7 days of storage respectively. According to Ağaoğlu et al. (2007) *P. aeruginosa* is among the most resistant bacteria toward the EOs, while it has susceptibility to cinnamon.

In summary EOs containing aldehydes or phenols, such as cinnamaldehyde, citral, carvacrol, eugenol or thymol at high concentration showed the highest antibacterial activity. The EOs containing terpene alcohols had the second highest level of antimicrobial activity. Other EOs, containing ketones or esters, such as β-myrcene, α-thujone had much weaker antimicrobial activity. Volatile oils containing terpene hydrocarbons were mostly inactive (Bassolé and Juliani, 2012).

Antibiotics and synthetic chemical products are widely used as a treatment to inhibit pathogenic bacteria and extending the shelf-life of foods (Hernández-Ochoa et al., 2011). As chemical

additives might have harmful effects in long-term usage, consumers prefer to have natural preservatives in foods to have healthier and less processed food with minimum synthetic additives in it (Mello da Silveira et al., 2014). Hence the food companies tend to replace the synthetic antimicrobial agents partially or completely with natural antimicrobials, to prepare natural food.

For the second part of the project a combination of Chinese cinnamon and Cinnamon bark was chosen from the first part to be combined with other antimicrobial agents and the antilisterial activity of 16 formulations were evaluated on fresh pork sausages. The results showed that all the antimicrobial formulations tested were effective against *L. monocytogenes* during short-term storage (7 days). In addition, the selected formulations (F1 and F9) were organoleptically accepted.

Each single preservation method is not efficient enough to achieve all the goals such as microbial safety, having naturally tasting food with less chemical preservation with reasonable long shelf life. Using synthesis preservatives as a direct method against pathogens for a long time could cause harmful effect such as carcinogenic effects on humans. Hence, Hurdle technology or combining different preservation methods (either different technologies and/or different antimicrobial agents) was used in this study as it enables us to use antimicrobials at low concentrations and improve food safety with no negative effect on the organoleptic properties. For instance, high concentrations of EOs are needed to inhibit bacteria while it could exceed the acceptable flavour threshold. In order to keep the high organoleptic quality of sausages, EOs were combined with nitrite, organic acid salts and nisin. Nutrients in food system which are not in growth media may promote cellular repair and can enhance the bacterial resistance to antimicrobial agents. That is why we need more concentration of antimicrobials in food system

compare to culture medium (Cui et al., 2010). Hence, it is necessary to combine antimicrobial compounds together to reduce their concentration and improve their efficiency.

As our food model was sausage which is a complex food system, it was necessary to mix the antimicrobial agents with it. In this study the antimicrobial compounds were encapsulated in edible polymer and the encapsulated antimicrobial compounds were mixed with sausages. The use of encapsulation technology can improve the adsorption of components. Incompatibility of additives is one of the concerns of food companies. For example the hydrophobic character of EOs cause phase separation in food with high water content (Quirós-Sauceda et al., 2014). But encapsulation can increase the physical and chemical stability and enables the lipophilic component to be dispersed in the aqueous phase. Furthermore, it protects the components from being degraded by food ingredients (Weiss et al., 2009).

Alginate as a polymer was used in this study to entrap the antimicrobial compounds and disperse them into the food system. The use of this polymer improves the viscosity and the binding reaction with water (Huq et al., 2013). Alginate is a natural and most widely used material for biopolymeric film. In fact it is an anionic polysaccharide composed of mannuronic acid and guluronic acid residues, which is derived from marine plants (seaweed). Alginate has many unique colloidal properties such as thickening, stabilizing, suspending, film forming, gel producing, and emulsion stabilizing which make it a promising biopolymer to be used as a film component (Huq et al., 2012).

Essential oils (EOs) as natural antimicrobial agents are promising compounds to be used as one of the factors in Hurdle technology. EOs mainly inhibit the bacteria by permeabilizing the cell membrane. So having EOs in an antimicrobial formulation can make bacteria more sensitive to

other antimicrobial agents or other processing technologies. For instance, it can render bacteria more sensitive to increase temperature (Cui, Li, et al., 2011).

Food ingredients can change the antimicrobial efficiency of EOs. For instance, Gutierrez et al. (2009) has demonstrated the positive effect of acidic pH (5) and simple sugars on efficiency of EOs. However, the high amount of fat can form a protective coating preventing the bacteria from being degraded by antimicrobial agents. Also, the nutrients in the food matrix might degrade the EOs (Zhang et al., 2009). So, the interaction between food ingredients and EOs should be considered (Cui et al., 2010).

EOs can work properly on lean meat compared to medium lean because EOs dissolve in the lipid phase while the bacteria are in the aqueous phase. So, EOs would be less effective against microorganisms in medium lean meat (Hernández-Ochoa et al., 2011; Rasooli et al., 2006). In this study lean ground pork was used as a food model.

According to our results, the combination of Chinese cinnamon and Cinnamon bark showed the linear negative effect with $P < 0.002$ while the linear effect of nitrite, nisin and organic acid salts are not important in the model due to their P values which were higher than 0.3. Also this means that combination of EOs is the most important factor in the model. This combination of EOs at 0.05% didn't change the sensorial properties of sausages. Our results are in accordance with other studies. It was reported that EOs have antimicrobial activity against pathogenic bacteria at the range of 0.05-0.1% in the food system (Ceylan and Fung, 2004). The EOs can also be used as preservatives at the concentration of 0.02-0.05% in the food system without changing the organoleptic properties of the food (Cui et al., 2010).

According to Burt (2004) cinnamaldehyde was stable after a treatment of 30 minutes at 200 °C. Being heat stable is another benefit for cinnamaldehyde. It could be interesting for restaurants where food is prepared in advance. Combining EOs with other preservatives would be an effective method to prevent microbial growth. However, their effectiveness depends on the pH, the temperature, the amount of oxygen, their concentration and indeed the presence of active compounds (Tajkarimi et al., 2010).

The different antimicrobial activities of EOs in combination with sodium nitrite was observed in growth media and ground pork which could be due to nutrients in food that promote cellular repair and increased the resistance of tested bacteria (Cui et al., 2010).

The combination of nitrite and nisin illustrated high antilisterial activity. Nitrite under the form of NO is used as a preservative is also implicated in the bright pink colouring of cured meat (Honikel, 2008). Nitrite is rather toxic for humans in comparison to nitrate. According to Honikel (2008), the permitted concentration of nitrite is 100 to 200 ppm while the permitted concentration of nitrate is below 500 ppm (Nyachuba et al., 2007). The residual nitrite level decreases after cooking and during storage time due to conversion to nitrate or nitric oxide or by binding with ingredients found in food (Nyachuba et al., 2007). According to the results obtained in this study, low concentrations of nitrite (100 ppm) in formulations were as effective as high nitrite concentrations (200 ppm). The formulation containing low concentrations of nitrite could also reduce the growth of *L. monocytogenes* during storage time. In addition, our results showed that nitrite at the concentration of 100 and 200 ppm could significantly inhibit the growth of *L. monocytogenes* and the antimicrobial efficiency of these two concentrations was not significantly different from each other (APPENDICES-1). Nyachuba et al. (2007) showed that sodium nitrite can significantly reduce the growth rate of *L. monocytogenes* and induce injury.

Myers et al. (2013) also revealed that nitrite doesn't stop the growth of *L. monocytogenes*; in fact it slows the growth of this bacterium.

The combination of nitrite and organic acid salts showed better activity than each of them individually. Nyachuba et al. (2007) reported that the antimicrobial activity of nitrite will be promoted in combination with other factors.

Nisin was another antimicrobial agent which was used in Hurdle technology. It is heat stable and it can even be used at very low concentrations depending on the target bacteria (Zacharof and Lovitt, 2012). Since nisin is mostly effective against Gram-positive bacteria, in order to improve the efficiency either against Gram-positive or Gram-negative, nisin can be used in Hurdle technology. For instance, if nisin is used in combination with another technology like heat treatment or with EO, it could express an additive effect since they could disintegrate the protective outer membrane and make the bacteria more sensitive to nisin (García et al., 2010; Solomakos et al., 2008a). Our results illustrated that the combination of nisin and organic acid salts (potassium lactate and sodium acetate) inhibited the growth of *L. monocytogenes* more effectively than each of the compounds alone. Zacharof and Lovitt (2012) reported the same trend as they showed nisin alone cannot ensure the food safety. It has to be combined with other technologies or other antimicrobial agents such as sodium acetate or sodium lactate. Another advantage of adding lactate is to stabilize the pH of vacuum-packaged beef (Crist et al., 2014). Apostolidis et al. (2008) confirmed the increase in antimicrobial activity against *L. monocytogenes* by combining the essential oils with salt of organic acid salts.

It is reported that organic acid salts are able to inhibit the growth of *L. monocytogenes* at 4°C. Combination of 2.5% lactate with 0.25% acetate, has an effect against *Listeria* and could

promote the safety of vacuum-packed ready-to-eat cooked meat products stored for 4–6 weeks (Blom et al., 1997).

Multiple barrier technology or Hurdle technology helps to combine several antimicrobial factors at their sub-inhibitory concentrations and/or combine them with other technologies for preservation and they can control the growth of microorganisms (Manju et al., 2007). In this study with the formulation, we have developed the combinations of EOs, salts of organic acid salts (potassium lactate and sodium acetate), nisin and nitrite provide benefits for both food safety and human health which means this combination can be used as a natural multiple-barrier food preservatives.

CHAPTER 5: SYNTÈSE DU MÉMOIRE RÉDIGÉ EN FRANÇAIS

1. INTRODUCTION

Au cours des dernières années, plusieurs questions microbiologiques en matière de sécurité alimentaire sont apparues (Kotzekidou, 2013). Il y a environ 4 millions de maladies d'origine alimentaire au Canada ce qui provoque un fardeau économique d'environ 3,7 milliards de dollars par an (Nesbitt et al., 2014).

Les aliments peuvent être contaminés pendant la conservation, la manutention (préparation), et même après la cuisson en raison d'une mauvaise manipulation. Malgré les progrès récents des technologies de contrôle, le nombre de maladies d'origine alimentaire a augmenté au cours des dernières années. Une nouvelle technologie est donc nécessaire dans le but d'éliminer les bactéries pathogènes du système alimentaire et assurer ainsi la sécurité alimentaire.

En effet, les consommateurs demandent des produits de haute qualité dont la salubrité a été reste naturel. Ceci a conduit les entreprise alimentaires à utilise du produits naturels à des concentrations faibles dans le but d'évités tout changement organoleptique.

2. REVUE DE LA LITTÉRATURE

2.1. Microbiologie des viandes

La viande car ils apporteur tous les acides aminés et les minéraux nécessaires et les produits carnés ont une place importante dans l'alimentation des consommateurs. La viande hachée est un système alimentaire complexe et puisqu'il possède des glucides solubles, des protéines, des enzymes endogènes favorisant ainsi la croissance des bactéries. La courte durée de vie de la viande hachée nécessite de développer des technologies de conservation (Dave and Ghaly, 2011; Mello da Silveira et al., 2014; Zhou et al., 2010). *Escherichia coli* O157: H7, *Salmonella* et

L. monocytogenes sont parmi les micro-organismes les plus dangereux qui peuvent être retrouvés dans les viandes (Hernández-Ochoa et al., 2011). Les agents antimicrobiens sont ainsi nécessaires dans les viandes transformées afin de contrôler la croissance des micro-organismes indésirables (Tajkarimi et al., 2010).

Dans cette étude, la saucisse de porc frais a été utilisée principalement comme un modèle alimentaire. L'activité antimicrobienne *in vitro* et *in situ* été déterminée contre cinq agents pathogènes d'origine alimentaire et des bactéries d'altération.

2.1.1. Listeria monocytogenes

Listeria est un pathogène d'origine alimentaire, Gram positif, causant la listériose. Cette bactérie est l'une des plus grandes préoccupations de santé publique, car elle peut être trouvée partout dans la nature (Ramaswamy et al., 2007).

Alors que la plupart des bactéries ne peuvent pas se développer en dessous de 4 °C, *Listeria* se développe dans une vaste gamme de températures (-4 °C et 50 °C) (Cammack et al., 1999; Kotzekidou, 2013; Ramaswamy et al., 2007). L'infection se fait lors de la consommation d'aliments contaminés, la viande crue principalement. Les bactéries peuvent affecter le système nerveux central causent des maladies graves telles que la méningite (Ramaswamy et al., 2007). En fait, 20-30% des infections ont un risque plus élevé et pourrait être fatales (Ramaswamy et al., 2007).

2.1.2. Staphylococcus aureus

S. aureus est une bactérie Gram-positif coccique qui est fréquemment trouvée dans les voies respiratoires humaines et qui peut se développer dans une large gamme de températures (6-48 °C). La consommation d'aliments contaminés par des toxines de staphylocoques cause une intoxication alimentaire staphylococcique qui est une maladie gastro-intestinale. Aux États-Unis,

environ 1200 décès dus à une intoxication alimentaire staphylococcique sont signalés chaque année (Mead et al., 1999). *S. aureus* peut être résistantes aux antibiotiques tels que la pénicilline et la méthicilline (Solorzano-Santos and Miranda-Novales, 2012).

2.1.3. Escherichia coli

E. coli est une bactérie Gram-négatif qu'il retrouve fréquemment dans l'intestin grêle des organismes à sang chaud. *E. coli* O157: H7 est un pathogène important qui provoque des symptômes sévères. La consommation de viande de bœuf hachée insuffisamment cuite et contaminés par *E. coli* cause généralement des maux d'estomac, mais peut aussi causer insuffisance rénale, ce qui peut conduire à la mort. Il semble que *E. coli* pourrait facilement devenir résistant aux antibiotiques (Solorzano-Santos and Miranda-Novales, 2012). Près de 25 000 cas par an estimés aux États-Unis (http://www.about-ecoli.com)

2.1.4. *Salmonella* Typhimurium

S. Typhimurium est une bactérie Gram négatif pathogène qui peut être trouvée trouve dans les intestins des animaux et des oiseaux. L'infection est généralement causée par l'ingestion de viande crue ou insuffisamment cuite, de volaille, d'œufs (Kotzekidou, 2013).

Les aliments contaminés par la bactérie *Salmonella* peut causer la salmonellose (Bajpai et al., 2012). L'abus d'antibiotiques par l'industrie alimentaire est à l'origine de la résistance des bactéries. Il est estimé que de 2 à 4 millions de cas de salmonellose surviennent chaque année aux États-Unis.

2.1.5. Pseudomonas aeruginosa

P. aeruginosa est une bactérie Gram négatif, coccobacillus, aérobie et anaérobie facultatif. Cette bactérie trouve dans le sol et l'eau (Neves et al., 2014). Cette bactérie peut rapidement devenir résistante aux agents antibactériennes (Solorzano-Santos and Miranda-Novales, 2012). Lorsque

le système de refroidissement n'est pas adéquat, la détérioration des aliments devient plus importante (Arslan et al., 2011). Chaque année aux États-Unis, autour de 51 000 infections à *P. aeruginosa* se produisent, dont environ 13% d'entre eux sont multi résistantes et provoquent environ 400 décès.

(http://www.cdc.gov/hai/organisms/pseudomonas.html)

3. Agents antimicrobiens qui ont été utilisés dans cette étude

Les agents antimicrobiens utilisés pour inhiber la croissance des bactéries cibles sont les huiles essentielles (HE), la nisine, le nitrite, le sel de l'acide organique.

3.1. Le nitrite

Le nitrite est un agent antimicrobien alimentaire capable de prolonger la durée de conservation de la viande. Il contribue également à la stabilité de la couleur tout en améliorant la qualité sensorielle en leur attribuant, une texture et une saveur unique à la viande (Cui et al., 2010; Sindelar and Milkowski, 2011).

Il peut y avoir production de nitrosamines en raison du pH acide de l'estomac (Davidson et al., 2010; Honikel, 2008). D'où l'utilisation de nitrite est strictement réglementée. La plus forte concentration de sel de nitrite dans les aliments devrait être inférieure à 200 ppm (Cui et al., 2010). L'équilibre entre les risques et les avantages pour les conservateurs alimentaires est toujours indispensable (Davidson et al., 2010).

3.2. La nisine

La nisine est un polypeptide antibactérien de synthèse ribosomique avec des résidus d'acides 34 aminés est utilisé comme agent de conservation alimentaire. La nisine est produite par des bactéries lactiques. Il s'agit d'un additif reconnu comme étant sans danger pour la consommation de l'homme (Zacharof and Lovitt, 2012).

La nisine a reconnu en tant que conservateurs alimentaires et actuellement il est largement approuvé utilisé dans plus de 50 pays. Il est efficace contre les bactéries pathogènes et d'altération des aliments ainsi que *S. aureus* et *L. monocytogenes* (Zacharof and Lovitt, 2012).

3.3. Sels acide organique

Les acides organiques et leurs sels sont utilisés comme conservateurs dans aliments pour prolonger la phase de latence de la prolifération microbienne provoquant ainsi un retard dans la croissance des bactéries. Par exemple la présence de sels d'acides organiques dans les saucisses augmente la durée de vie des saucisses (Crist et al., 2014; Ibrahim Sallam, 2007).

Les principaux sels d'acides organiques utilisés sont le lactate de potassium et l'actate de sodium. Ils sont capables d'inhiber la croissance de bactérie (Stekelenburg, 2003).

Ibrahim Sallam (2007) a montré, que l'acétate de sodium peut être utilisé comme conservateur son activité antimicrobienne est élevé car il a effet négative sur la croissance de diverses bactéries pathogènes. L'acétate de sodium est approuvé comme agent aromatisant (Ibrahim Sallam, 2007).

3.4. Les huiles essentielles

Au course de leur évaluation, les plantes ont produit de HE pour se défendre contre les prédateurs (insectes, champignons, etc) et les pathogènes microbiens (Bassolé and Juliani, 2012). Les Huiles essentielles sont principalement extraites des plantes dans les pays tropicaux ou méditerranéens (Bakkali et al., 2008).

Les HE ont été utilisées pour diverses raisons depuis l'antiquité (Bakkali et al., 2008; Porres-Martínez et al., 2013; Solorzano-Santos and Miranda-Novales, 2012). Environ 3000 HE sont connues et 300 d'entre elles sont utilisées commercialement dans les parfums, la dentisterie,

l'agriculture et les produits alimentaires. La cannelle, le clou de girofle, la moutarde, l'ail, le gingembre et la menthe sont traditionnellement utilisées dans les remèdes de santé dans les pays asiatiques (Tajkarimi et al., 2010). Les HE sont l'un des meilleurs candidats antimicrobiens pour l'utilisation comme agents de conservation dans le système alimentaire (Tajkarimi et al., 2010).

3.4.1. Les facteurs influcent sur les propriétés des HE

La densité de plantation, l'âge, le climat, la région, la composition du sol, la saison de récolte, les parties de la plante utilisées pour extraire les HE, et aussi la façon de distillation sont les facteurs qui peuvent affecter les propriétés d'une HE (Lacroix, 2007; McGimpsey et al., 1994; Oussalah et al., 2007).

3.4.2. Composés majeurs et mineurs

Il y a plus d'une soixante de composants individuels dans une huile essentielle. L'effet principal d'une HE est attribué à la molécule principale qui la compose. Cependant, les composés mineurs pourraient également avoir une activité synergique ou additive avec les plus importants (Bassolé and Juliani, 2012; Burt, 2004; Hyldgaard et al., 2012; Oussalah et al., 2007; Turgis et al., 2009).

3.4.3. Mécanisme d'HE

Le caractère lipophile des composants des HE contribué à leur effet antimicrobien. En effet, elles peuvent s'accumuler dans la bicouche lipidique de la membrane cellulaire, qui va subir une la perte d'ions et une diminution d'ATP, provoquent la mort cellulaire (Bakkali et al., 2008) (Oussalah et al., 2006; Quirós-Sauceda et al., 2014).

En outre, les HE peuvent endommager la chaîne de synthèse de matériaux génétiques (Hernández-Ochoa et al., 2011). En effet, certains composants des HE pourraient se lier à la protéine, empêcher l'activité de l'enzyme et provoquer la mort cellulaire (Lacroix, 2007).

3.4.4. HE dans les aliments

Bien que les HE ont le statut GRAS et montré l'effet antimicrobien prometteur, leur application est limitée en raison de leur goût et leur odeur forte

3.4.5. Interaction des HE avec la matrice alimentaire

Les ingrédients alimentaires peuvent influer sur l'efficacité de l'OT. Certaines études ont démontré l'effet négatif de la grande quantité de graisse sur l'efficacité d'HE (Celikel and Kavas, 2008).

3.4.6. HE dans les traitements combinés

La combinaison avec un autre composé peut modifier l'activité antimicrobienne d'HE. Par exemple une bactériocine comme la nisine, ou encore une autre HE. L'effet de ces composés sur l'activité antimicrobienne des HE pourrait être synergique, plus ou antagonistes.

4. TECHNIQUES POUR LA CONSERVATION

En général pour la conservation de la viande les méthodes utilisées peuvent être classées en trois principaux groupes: le contrôle de la température, le contrôle de l'humidité et l'effet direct sur les micro-organismes (Zhou et al., 2010).

4.1. Contrôle de la température

La température peut contrôler la croissance de bactéries ou de les éliminer, si elle est au-dessous ou au-dessus de la plage optimale pour la croissance bactérienne. Dans le cas de la viande fraîche, de la réfrigération a été traditionnellement utilisé comme une méthode de conservation (Zhou et al., 2010).

4.2. Contrôle de l'humidité

L'ajout de sel augmente la pression osmotique qui attire l'eau hors des micro-organismes, et ralentit le taux de processus d'oxydation (la concentration de NaCl devrait être autour de 20%) (Sindelar and Milkowski, 2011).

4.3. Processus d'inhibition qui a un effet direct sur les micro-organismes

Une autre technique pour la conservation des aliments est l'utilisation des agents antimicrobiens. Les agents antimicrobiens inhibent la croissance bactériennes soit en attaquant la membrane bactériennes ou en désactivent les enzymes essentiels ou par divers moyens.

4.3.1. Irradiation

L'irradiation gamma dans cette étude a été utilisée principalement pour stériliser les échantillons de viande. La radiothérapie peut tuer les micro-organismes et les virus en endommageant l'ADN et produisant des peroxydes, qui sont des agents oxydants très puissants dans les cellules.

Les rayonnements ionisants peuvent contrôler les micro-organismes sans augmenter la température de façon significative, de ce fait elle est également appelé pasteurisation à froid (Alighourchi et al., 2014). L'irradiation utilisé dans environ 56 pays (Alighourchi et al., 2014). Jebri et al. (2013) a montré que les rayonnements ionisants efficaces peuvent inactiver les microorganismes pathogènes dans de l'eau, la nourriture et les produits médicaux.

Avant chaque expérience *in situ*, toutes les saucisses ont été irradiées à une dose de 45 kGy à l'aide d'un irradiateur UC-15A (MDS Nordion International Inc., Kanata, Ontario, Canada) équipé d'une source ^{60}Cobalt.

http://www.inspection.gc.ca/food/information-for-consumers/fact-sheets/irradiation/eng/1332358607968/1332358680017

5. UTILISATION DE LA MEILLEURE TECHNOLOGIE (HURDLE TECHNOLGIE)

Pour avoir la sécurité sans affecter les propriétés organoleptiques des aliments, de nouvelles méthodes pour contrôle antimicrobiens (technologies des barrières) doivent être établi (Cui, Gabriel, et al., 2011). La technologie de multi variable (Hurdle technology) utilise la combinaison de facteurs de transformation des aliments non agressive pour obtenir l'une sécurité acceptable et les qualités sensorielles élevées (Cui, Gabriel, et al., 2011).

Pour conserver la viande fraiche, il est préférable de ne pas utiliser un traitement thermique et le remplacer par d'autres technologies telles que les technologies de multi variable (Zhou et al., 2010). Les technologies de conservation non agressives sont importantes pour les industries alimentaires modernes et en combinant ces processus la qualité organoleptique sera améliorée. Cette méthode de conservation est économie d'énergie, respectueux de l'environnement, organoleptiquement acceptable et surtout très efficace pour inhiber les pathogènes (Zhou et al., 2010).

6. ENCAPSULATION DANS UN POLYMÈRE COMESTIBLE

Pour prolonger la durée de vie, il est nécessaire d'améliorer la sécurité et stabilité microbiologique ce qui signifie qu'il devrait avoir un contrôle sur la croissance des bactéries. L'utilisation d'agents antimicrobiens naturels ou synthétiques a des limites. Ils répandront des odeurs anormales ou seront dégradées par des ingrédients alimentaires et perdront leurs activités en peu de temps (Quirós-Sauceda et al., 2014).

Un polymère comestible peut contenir des protéines, des polysaccharides et aussi des lipides. Les composés peuvent être ajoutés seul ou ensemble.

L’Alginate, a été utilisé en tant que polymère pour encapsuler les solutions antimicrobiennes et les disperser dans le système alimentaire. Il améliore également la viscosité et se lie à l'eau (Huq et al., 2013). L’encapsulation des agents antimicrobiens dans des polymères comestibles nous apporte des avantages notables:

6.1. Contrôle de la diffusion

En piégeant le composé antimicrobien on ralentit sa libération dans le produit. De cette façon, l'agent antimicrobien va durée pour un temps plus long et à la suite de cela, la durée de conservation des aliments serait étendu (Neetoo et al., 2010; Quirós-Sauceda et al., 2014). Le contrôle de la libération du composé encapsulé se fait par différents moyens tels que, la fusion, la diffusion, la dégradation ou la fracture des particules (Quirós-Sauceda et al., 2014).

6.2. Conserver le goût naturel

La libération du composé antimicrobien est contrôlée par l’encapsulation, ce qui réduit l’effet négatif de chaque composant sur l’aspect organoleptique (Neetoo et al., 2010; Quirós-Sauceda et al., 2014).

6.3. Promotion de la solubilité

La plupart des agents antimicrobiens inhibent les bactéries en endommageant leur membrane cellulaire, ou leurs enzymes. L’encapsulation améliore la solubilité des composés antimicrobiens et les rend disponibles dans l’intégralité de la matrice alimentaire (Neetoo et al., 2010; Quirós-Sauceda et al., 2014).

6.4. Préservation de la bioactivité

Il est indiqué qu’une forte concentration de lipides, de glucides et de CO_2 provoque une réduction de l'activité antimicrobienne des HE (de Oliveira et al., 2011). L’encapsulation des

agents antimicrobiens dans des polymères comestibles pourrait contrôler l'interaction entre les facteurs encapsulés et la matrice alimentaire.

7. DISCUSSION

Les résultats du premier article ont révélé que les HE comme le thym rouge, la bergamote Rouge, la Sarriette, la cannelle de Chine et l'écorce de cannelle étaient les plus efficaces *in vitro*. Comme les HE sont constituées de différents composants, il est difficile de trouver leur cible spécifique (Hyldgaard et al., 2012). Comme les HE ont des cellules cibles différentes, aucune résistance particulière n'a encore été signalée, ce qui peut expliquer le choix de ces molécules en tant que candidats prometteurs pour la conservation des aliments (Bakkali et al., 2008). Les HE perméabilisent la membrane cellulaire par solubilisation des lipides de cette dernière (Bakkali et al., 2008; Turgis et al., 2009).

Selon l'ensemble de nos résultats *in vitro*, les HE étaient généralement plus efficaces contre les bactéries Gram-positif (*L. monocytogenes* et *S. aureus*) que les bactéries ¸à Gram-négatif (*E. coli*, *S.* Typhimurium et *P. aeruginosa*). Cependant, parmi les bactéries à Gram-négatif, *E. coli* a montré une plus grande sensibilité aux HE testées. Le mécanisme d'inhibition des HE est différent chez les bactéries à Gram-positifs par rapport aux Gram-négatifs. Les HE ont, pour la plupart, des constituants hydrophobes qui perturbent la structure lipidique des bactéries à Gram-positives en s'accumulant dans la membrane et entraînent une perte de l'intégrité en ce qui concerne les bactéries à Gram-négatif, les HE pénètrent les bactéries en se liant à certaines protéines dans la membrane (Lacroix, 2007; Oussalah et al., 2006).

Dans notre étude, le thym rouge, la bergamote Rouge, la Sarriette, la cannelle de Chine et l'écorce de cannelle, les principaux composants sont respectivement le thymol 48.03%, la

carvacrol 48.21%, la carvacrol 26,8%, le Trans-cinnamaldéhyde 87,58% et le Trans-cinnamaldéhyde 40,71% étaient les plus efficaces dans le contrôle de la croissance des bactéries.

Selon Hyldgaard et al. (2012), les composants actifs des HE peuvent être classés en quatre groupes en fonction de leur structure chimique: les terpènes, les terpénoïdes, phénylpropènes, et autres. Parmi les HE que nous avons évalués, il y avait certains qui contiennent une grande quantité d'hydrocarbures tels que Melissa (β-caryophyllène 23,31%), le genévrier commun (α-pinène 75,61%), le sapin baumier (β-pinène 31,41%), le pin rouge (α pinène 49,49%), du pin blanc (α-pinène 29,82%), et Ajowan (γ-terpinène 36,40%). À l'exception d'Ajowan qui a montré une efficacité élevée contre les bactéries testées dans la diffusion sur gélose et la methode de micro-atmosphere, les autres ne montrent pas d'activité antimicrobienne élevée avec les méthodes testées. Généralement, les HE à haute teneur en hydrocarbures démontrent une faible activité antimicrobienne par rapport aux autres (Bassolé and Juliani, 2012). Les terpènes sont des hydrocarbures fabriqués à partir de 5 unités de carbone (isoprène) (Bakkali et al., 2008). Des terpènes comme l'α-pinène, β-pinène, le p-cymène, le γ-terpinène, et le β-caryophyllène ne possédant pas d'activité antimicrobienne (Hyldgaard et al., 2012).

Un autre composé actif des HE est un terpénoïde qui est terpénique contenant de l'oxygène et peut être subdivisé pour les alcools, les phénols, les aldéhydes, les éthers, les esters, les cétones. Les terpénoïdes sont actifs contre un large spectre de micro-organismes (Hyldgaard et al., 2012). Dans notre étude les HE de Sarriette, de bergamote Rouge et d'origan contiennent 26,8%, 48,21% et 21,01% de carvacrol respectivement en tant que composant principale. Winter savory et la bergamote Rouge ont montré une grande efficacité contre les bactéries testées (Gram-positif ou des Gram-négatifs), à l'exception de *P. aeruginosa* qui était la plus résistante à HE parmi nos bactéries testées, tandis que l'origan est principalement efficace contre *S. aureus*, *E. coli* et *S.*

Typhimurium. Le carvacrol est un phénol isoprénylique qui a une activité antimicrobienne, et qui est plus efficace à pH acide (4,0). Les enveloppes cellulaires sont la principale cible du carvacrol puisqu'il désintègre la membrane externe en modifiant la morphologie de la membrane cellulaire. Le carvacrol fait acidifier le cytoplasme par perméabilisations de la membrane ce qui entraine la sortie de protons et de potassium et affectant ainsi le gradient d'ions à travers la membrane (Oussalah et al., 2006). En fait, le groupe hydroxyle libre et les échanges de protons sont la principale raison de l'activité bactéricide de carvacrol (Ait-Ouazzou et al., 2013).

Grâce à leur groupe hydroxyle, les composés phénoliques tels que le thymol et le carvacrol jouent un rôle important dans l'inhibition des bactéries (Jayasena and Jo, 2013). En outre, nos résultats ont montré que l'HE de Thym commun, de Thym rouge, et d'Ajowan contenant respectivement 34,70%, 48,03% et 32,35% de thymol pourraient inhiber l'efficacité des bactéries à l'exception de thym commun dans le test du bouillon de micro-dilution. La présence de thymol perturbant la membrane cellulaire et interagirait avec les protéines de la membrane (Hyldgaard et al., 2012; Oussalah et al., 2006).

Les HE de bergamote sauvage et de palmarosa, à fortement concentrées eu géraniol (91,71% et 80,14% respectivement) ont montré une activité antimicrobienne d'amende contre les bactéries testées, mais pas dans la méthode de diffusion sur gélose. La combinaison de bergamote sauvage avec d'autres HE comme la bergamote rouge, la cannelle de Chine et l'écorce de cannelle a généralement montré une efficacité additive. Le géraniol peut moduler la résistance aux médicaments dans plusieurs bactéries à Gram-négatif (Solorzano-Santos and Miranda-Novales, 2012). Il existe de nombreuses huiles essentielles qui ont au moins un noyau benzénique avec un groupe fonctionnel hydroxyle et qui sont connus en tant que composés phénoliques (Ebrahimabadi et al., 2010). La présence et l'emplacement de groupes hydroxyles dans les HE,

déterminent l'efficacité antimicrobienne des HE (Lacroix, 2007). Le Groupe hydroxyle peut désactiver les enzymes et affecte des acides gras et des phospholipides, rendant la membrane plus perméable (Hernández-Ochoa et al., 2011).

Il a été montré que les composés non phénoliques ont aussi un effet antimicrobien. Notre étude a démontré que 83 à 95% de gousse de l'eugénol avait une forte activité antimicrobienne avec toutes les méthodes. En outre, selon les résultats de « checkerboard », la combinaison de clou de girofle avec la bergamote rouge a montré un effet additif contre *L. monocytogenes*, *E. coli* et *S.* Typhimurium.

Il a été prouvé que l'eugénol inhibent l'activité de l'ATPase entrainant la mort cellulaire dû à l'arrêt de production d'énergie (Hyldgaard et al., 2012). Le troisième type de composant actif de l'HE est le Phénylpropanoïdes. L'eugénol, le cinnamaldéhyde et la vanilline sont quelques uns des phénylpropènes (Hyldgaard et al., 2012). Leur activité dépend du type et du nombre de substituants sur le cycle aromatique, du type de bactéries, du support, et de la température (Hyldgaard et al., 2012).

La Cannelle de Chine et l'écorce de cannelle, dans notre étude, avec le Trans-cinnamaldéhyde comme composant principal (87,58% et 40,71% respectivement) ont été parmi les HE les plus efficaces dans toutes les méthodes *in vitro*, et la combinaison de ces deux huiles a été choisie car elle montre un effet additif contre toutes les bactéries testées dans la méthode de « checkerboard ». Cette combinaison pourrait inhiber la croissance de toutes les bactéries testées autour de 0,16 (*L. monocytogenes*) à 0,52 (*E. coli*) log *in situ* (viande de porc haché) jusqu'à 7 jours de stockage.

Les groupes aldéhyde peuvent faire une liasion croisé avec l'ADN et les protéines à travers des groupes amines (Lacroix, 2007). Le cinnamaldéhyde, à faible concentration, pourrait inhiber certains enzymes qui ne sont pas nécessaire à la cellule. À forte concentration, il peut atteindre le périplasme et inhiber l'ATPase et à une concentration létale, il perturbe la membrane (Hyldgaard et al., 2012). L'huile de cannelle est l'un des HE qui a un effet à la fois contre la culture planctonique et contre des biofilm de bactéries telles que *S. epidermis* (Solorzano-Santos and Miranda-Novales, 2012).

Parmi toutes les bactéries testées dans notre étude, *P. aeruginosa* avait le plus de résistance. Cependant, nos résultats illustrent la grande efficacité de la combinaison de la cannelle de chine et de l'écorce de cannelle dans le contrôle de la croissance de *P. aeruginosa* à la fois *in vitro* et *in situ*. Dans le modèle de la viande, la combinaison des cannelles réduisent la bactérie de 0,62, 0,33, et 0,23 log après 1, 4, et 7 jours de conservation, respectivement. Selon Ağaoğlu et al. (2007) *P. aeruginosa* est parmi les bactéries les plus résistantes aux HE, alors qu'elle est sensible à la cannelle.

Comme les additifs chimiques peuvent avoir des effets nocifs dans l'utilisation à long terme, les consommateurs préfèrent avoir des conservateurs naturels dans les aliments (Mello da Silveira et al. 2014). Par conséquent, les entreprises alimentaires veulent remplacer les agents antimicrobiens synthétiques partiellement ou totalement par des antimicrobiens naturels.

Pour la deuxième partie du projet, la combiné de cannelle de Chine et de l'écorce de cannelle a été choisi à partir de la première partie pour être combinaison avec du nitrite, la nisine et des sels d'acides organiques. L'activité antimicrobienne des 16 formulations a été évaluée sur des saucisses de porc frais. Les résultats montrent que toutes les formulations antimicrobiennes

étaient efficaces contre *L. monocytogenes* après 7 jours de conservation. En outre, les résultats ont montré que les formulations sélectionnées (F1 et F9) ont été organoleptiquement acceptés.

La technologie des barrières ou la combinaison de différentes méthodes de conservation (soit différentes technologies et / ou différents agents antimicrobiens) a été utilisée dans cette étude car elle nous permet d'utiliser des antimicrobiens à de faibles concentrations et garantie d'avoir une sécurité alimentaire sans effets négatifs sur les propriétés organoleptiques. Les éléments nutritifs présents dans le produit alimentaire, et absents dans les milieux de culture, peuvent favoriser la réparation cellulaire. Pour obtenir la même efficacité, les agents antimicrobiens doivent donc être utilisés à des concentrations plus élevées que celles *in vitro* (Cui et al., 2010). Par conséquent, il est nécessaire de combiner des composés antimicrobiens ensemble afin de réduire leur concentration, éviter tout effet négatif sur les propriétés organoleptiques des aliments et d'obtenir un rendement encore plus élevé.

Notre modèle alimentaire étant la saucisse, un système alimentaire complexe, il est nécessaire de mélanger les agents antimicrobiens avec cette dernière. C'est pourquoi les antimicrobiens ont été encapsulés dans un polymère comestible et ces formulations antimicrobiennes encapsulés ont été mélangées avec viande des saucisses. L'encapsulation améliore l'absorption et adsorption des composants et peut également augmenter la stabilité physique et chimique en permettant au composant lipophile d'être dispersé dans la phase aqueuse. En outre, il protège ces composés bioactifs d'une éventuelle dégradation par les ingrédients alimentaires (Weiss et al., 2009).

Les HE inhibent les bactéries principalement en perméabilisant leurs membranes cellulaires. la présence de l'HE dans la formulation antimicrobienne peut rendre les bactéries plus sensibles qu'avec d'autres agents antimicrobiens ou d'autres technologies de traitement (Cui et al., 2010).

Les ingrédients de l'aliment peuvent modifier l'efficacité antimicrobienne des HE. En effet la grande quantité de graisse peut former une couche protectrice autour des bactéries, qui empêche la dégradation par les agents antimicrobiens. En outre, les éléments nutritifs de la matrice alimentaire peuvent aider la réparation des bactéries endommagées et peuvent dégrader les HE (Zhang et al., 2009). Dans notre étude, nous avons utilisé du porc haché maigre afin de réduire les effets dus à la présence de graisse.

Nos résultats ont montré que la combinaison de cannelle de Chine et de l'écorce de cannelle a montré un effet linéaire sur la croissance de *L. monocytogenes*. Cette combinaison d'HE à 0,05% n'a pas modifié les propriétés sensorielles des saucisses. Nos résultats sont ainsi en accord avec d'autres études.

Tajkarimi et al. (2010) ont démontré que les HE ont une activité antimicrobienne contre les bactéries pathogènes dans la plage de 0,05 à 0,1% dans le système alimentaire. Ils peuvent être utilisés comme un agent de conservation à la concentration de 0,02 à 0,05% dans les aliments sans modifier les propriétés organoleptiques de celui ci (Cui et al., 2010).

Selon Burt (2004) le cinnamaldéhyde était stable après 30 minutes à 200 ° C. Cette stabilité face à la chaleur pourrait être intéressant pour les restaurants où ils cuisent et ne servent pas immédiatement.

La combinaison des HE avec d'autres agents de conservation serait suffisamment efficace. Leur efficacité dépend du pH, de la température, de la quantité d'oxygène, de leur concentration et de l'effet de la présence des composés actifs (Tajkarimi et al., 2010).

On a observé une différence dans l'activité antimicrobienne des HE et du nitrite de sodium dans les milieux de croissance et dans le porc haché. Cela pourrait être dû à des éléments nutritifs dans

les aliments qui favorisent la réparation cellulaire et causent moins de sensibilité des bactéries testées (Cui et al., 2010).

La combinaison de nitrite et de nisine ont illustré une activité antilisterial élevé. Le $NaNO_2$ est utilisé comme agent de conservation mais aussi peux fixer la couleur rose vif de la viande (Honikel, 2008). La concentration autorisée de nitrite est de 100 à 200 ppm (Nyachuba et al., 2007). Les résultats indiquent qu'une faible concentration de nitrite (100 ppm) dans des formulations était aussi efficace qu'une concentration élevée de nitrite (200 ppm) pour la formulation, à faible concentration, peut également réduire la croissance de *L. monocytogenes* pendant toute la durée de stockage. Cependant, les effets des deux concentrations de nitrite (100 et 200 ppm) ne sont pas significativement différents (ANNEXES-1). Des études ont montré que le nitrite de sodium peut réduire de façon significative le taux de croissance de *L. monocytogenes* (Nyachuba et al., 2007). La littérature a révélé que le nitrite n'arrête pas la croissance de *L. monocytogenes* mais provoque un ralentissement de croissance de cette bactérie (Myers et al., 2013).

La combinaison de nitrite et les sels d'acides organiques a montré une meilleure activité que chacun d'entre eux individuellement, indiquant un effet synergique. Nyachuba et al. (2007) ont indiqué que l'activité antimicrobienne du nitrite sera augmentée en combinaison avec d'autres facteurs.

La nisine est stable à la chaleur et peut même être utilisée à des concentrations très faibles selon bactérie cible (Zacharof and Lovitt, 2012). Si la nisine est combinée avec un autre agent qui déstabilise la membrane cellulaire (comme les HE), un effet additif pourrait être observé. En effet, la membrane externe des bactéries pourrait être désintégrée, rendant les bactéries plus sensibles à la nisine (García et al., 2010; Solomakos et al., 2008a). Nos résultats ont montré que

la combinaison de la nisine et des sels d'acides organiques (lactate de potassium et acétate de sodium) a inhibé la croissance de *L. monocytogenes* de manière plus efficace que chacun des composés seul. Zacharof et Lovitt, (2012) ont rapporté que la nisine seule ne peut pas assurer la sécurité alimentaire, et doit être combiné avec d'autres technologies ou d'autres agents antimicrobiens tels que l'acétate de sodium ou de lactate de sodium (Zacharof and Lovitt, 2012). D'autres études confirment l'augmentation de l'activité antimicrobienne contre *L. monocytogenes* en combinant les huiles essentielles avec un sel d'acides organiques (Apostolidis et al., 2008).

Des études ont démontré que les sels d'acides organiques sont capables d'inhiber la croissance de *L. monocytogenes* à 4 °C (Blom et al., 1997).

8. CONCLUSION

Pour résumer, les HE composés d'aldéhydes ou de phénols, comme le cinnamaldéhyde, le citral, le carvacrol, l'eugénol ou le thymol à une concentration élevée (comme principal composé) ont démontré l'activité antibactérienne la plus élevée. Les HE contenant des alcools terpéniques sont dans un deuxième niveau d'activité antimicrobienne. D'autres HE, avec des cétones ou des esters, tels que les β-myrcène, α-thujone ont une activité beaucoup plus faible. A la fin on trouve les huiles volatiles contenant des hydrocarbures terpéniques qui sont souvent inactives (Bassolé and Juliani, 2012).

La technologie de barrière multiple ou la technologie des barrières nous aident à combiner plusieurs facteurs antimicrobiens à leurs concentrations sub-inhibitrices et / ou les combiner avec

d'autres technologies de conservation afin qu'ils puissent contrôler la croissance des micro-organismes (Manju et al., 2007). Notre formulation, qui est une combinaison d'HE, de sels d'acides organiques (lactate de potassium et acétate de sodium), de nisine et de nitrite offre des avantages à la fois pour la sécurité alimentaire et la santé humaine, ce qui signifie que cette combinaison peut être utilisée comme un produit naturel de conservation des aliments à barrières multiples.

BIBLIOGRAPHIE

1. Abdollahzadeh, E., Rezaei, M., Hosseini, H., 2014. Antibacterial activity of plant essential oils and extracts: The role of thyme essential oil, nisin, and their combination to control *Listeria monocytogenes* inoculated in minced fish meat. Food Control 35, 177-183.
2. Ağaoğlu, S., Dostbil, N., Alemdar, S., 2007. Antimicrobial activity of some spices used in the meat industry. Bull Vet Inst Pulawy 51, 53-57.
3. Ait-Ouazzou, A., Espina, L., Gelaw, T.K., de Lamo-Castellvi, S., Pagan, R., Garcia-Gonzalo, D., 2013. New insights in mechanisms of bacterial inactivation by carvacrol. Journal of Applied Microbiology 114, 173-185.
4. Alighourchi, H., Barzegar, M., Sahari, M.A., Abbasi, S., 2014. The effects of sonication and gamma irradiation on the inactivation of *Escherichia coli* and *Saccharomyces cerevisiae* in pomegranate juice. Iranian Journal of Microbiology 6, 51-58.
5. Apostolidis, E., Kwon, Y.I., Shetty, K., 2008. Inhibition of Listeria monocytogenes by oregano, cranberry and sodium lactate combination in broth and cooked ground beef systems and likely mode of action through proline metabolism. International Journal of Food Microbiology 128, 317-324.
6. Arslan, S., Eyi, A., Özdemir, F., 2011. Spoilage potentials and antimicrobial resistance of *Pseudomonas* spp. isolated from cheeses. Journal of dairy science 94, 5851-5856.
7. Bajpai, V.K., Baek, K.-H., Kang, S.C., 2012. Control of *Salmonella* in foods by using essential oils: A review. Food Research International 45, 722-734.
8. Bakkali, F., Averbeck, S., Averbeck, D., Idaomar, M., 2008. Biological effects of essential oils – A review. Food and Chemical Toxicology 46, 446-475.

9. Bassolé, I.H.N., Juliani, H.R., 2012. Essential Oils in Combination and Their Antimicrobial Properties. Molecules 17, 3989-4006.
10. Bezerra, M.A., Santelli, R.E., Oliveira, E.P., Villar, L.S., Escaleira, L.A., 2008. Response surface methodology (RSM) as a tool for optimization in analytical chemistry. Talanta 76, 965-977.
11. Blom, H., Nerbrink, E., Dainty, R., Hagtvedt, T., Borch, E., Nissen, H., Nesbakken, T., 1997. Addition of 2.5% lactate and 0.25% acetate controls growth of *Listeria monocytogenes* in vacuum-packed, sensory-acceptable servelat sausage and cooked ham stored at 4°C. International Journal of Food Microbiology 38, 71-76.
12. Burt, S., 2004. Essential oils: their antibacterial properties and potential applications in foods—a review. International Journal of Food Microbiology 94, 223-253.
13. Cammack, R., Joannou, C.L., Cui, X.-Y., Torres Martinez, C., Maraj, S.R., Hughes, M.N., 1999. Nitrite and nitrosyl compounds in food preservation. Biochimica et Biophysica Acta (BBA) - Bioenergetics 1411, 475-488.
14. Cardiet, G., Fuzeau, B., Barreau, C., Fleurat-Lessard, F., 2012. Contact and fumigant toxicity of some essential oil constituents against a grain insect pest Sitophilus oryzae and two fungi, Aspergillus westerdijkiae and Fusarium graminearum. Journal of Pest Science 85, 351-358.
15. Celikel, N., Kavas, G., 2008. Antimicrobial properties of some essential oils against some pathogenic microorganisms. Czech journal of food sciences 26, 174-181.
16. Ceylan, E., Fung, D.Y., 2004. Antimicrobial activity of spices. Journal of Rapid Methods & Automation in Microbiology 12, 1-55.

17. Crist, C.A., Williams, J.B., Schilling, M.W., Hood, A.F., Smith, B.S., Campano, S.G., 2014. Impact of sodium lactate and vinegar derivatives on the quality of fresh Italian pork sausage links. Meat Science 96, 1509-1516.
18. Cueva, C., Moreno-Arribas, M.V., Bartolome, B., Salazar, O., Vicente, M.F., Bills, G.F., 2011. Antibiosis of vineyard ecosystem fungi against food-borne microorganisms. Res Microbiol 162, 1043-1051.
19. Cui, H., Gabriel, A.A., Nakano, H., 2010. Antimicrobial efficacies of plant extracts and sodium nitrite against *Clostridium botulinum*. Food Control 21, 1030-1036.
20. Cui, H., Gabriel, A.A., Nakano, H., 2011. Heat-sensitizing effects of plant extracts on *Clostridium spp*. spores. Food Control 22, 99-104.
21. Cui, P., Li, X., Man, Y., Fang, Z., 2011. Heat transfer analysis of pile geothermal heat exchangers with spiral coils. Applied Energy 88, 4113-4119.
22. Dave, D., Ghaly, A.E., 2011. Meat spoilage mechanisms and preservation techniques: A critical review. American Journal of Agricultural and Biological Sciences 6, 486.
23. Davidson, P.M., Sofos, J.N., Branen, A.L., 2010. Antimicrobials in food. CRC Press.
24. de Oliveira, T.L.C., de Araújo Soares, R., Ramos, E.M., das Graças Cardoso, M., Alves, E., Piccoli, R.H., 2011. Antimicrobial activity of Satureja montana L. essential oil against *Clostridium perfringens* type A inoculated in mortadella-type sausages formulated with different levels of sodium nitrite. International Journal of Food Microbiology 144, 546-555.
25. Devi, K.P., Nisha, S.A., Sakthivel, R., Pandian, S.K., 2010. Eugenol (an essential oil of clove) acts as an antibacterial agent against *Salmonella typhi* by disrupting the cellular membrane. Journal of Ethnopharmacology 130, 107-115.

26. Devlieghere, F., Vermeulen, A., Debevere, J., 2004. Chitosan: antimicrobial activity, interactions with food components and applicability as a coating on fruit and vegetables. Food Microbiology 21, 703-714.
27. Dorman, H.J., Deans, S.G., 2000. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. Journal of Applied Microbiology 88, 308-316.
28. Dussault, D., Caillet, S., Le Tien, C., Lacroix, M., 2009. Effect of gamma-irradiation on membrane fatty acids and peptidoglycan's muropeptides of Pantoea agglomerans, a plant pathogen. Journal of Applied Microbiology 106, 1033-1040.
29. Dussault, D., Vu, K.D., Lacroix, M., 2014. In vitro evaluation of antimicrobial activities of various commercial essential oils, oleoresin and pure compounds against food pathogens and application in ham. Meat Science 96, 514-520.
30. Ebrahimabadi, A.H., Djafari-Bidgoli, Z., Mazoochi, A., Kashi, F.J., Batooli, H., 2010. Essential oils composition, antioxidant and antimicrobial activity of the leaves and flowers of *Chaerophyllum macropodum* Boiss. Food Control 21, 1173-1178.
31. Fratianni, F., De Martino, L., Melone, A., De Feo, V., Coppola, R., Nazzaro, F., 2010. Preservation of chicken breast meat treated with thyme and balm essential oils. Journal of Food Science 75, M528-535.
32. García, P., Rodríguez, L., Rodríguez, A., Martínez, B., 2010. Food biopreservation: promising strategies using bacteriocins, bacteriophages and endolysins. Trends in Food Science & Technology 21, 373-382.
33. Gaulin, C., Nguon, S., Leblanc, M.A., Ramsay, D., Roy, S., 2013. Multiple outbreaks of gastroenteritis that were associated with 16 funerals and a unique caterer and spanned 6 days, 2011, Quebec, Canada. Journal of Food Protection 76, 1582-1589.

34. Gill, A.O., Delaquis, P., Russo, P., Holley, R.A., 2002. Evaluation of antilisterial action of cilantro oil on vacuum packed ham. International Journal of Food Microbiology 73, 83-92.
35. Gill, A.O., Holley, R.A., 2004. Mechanisms of bactericidal action of cinnamaldehyde against *Listeria monocytogenes* and of eugenol against *L. monocytogenes* and *Lactobacillus sakei*. Applied and Environtal Microbiology 70, 5750-5755.
36. Goñi, P., López, P., Sánchez, C., Gómez-Lus, R., Becerril, R., Nerín, C., 2009. Antimicrobial activity in the vapour phase of a combination of cinnamon and clove essential oils. Food Chemistry 116, 982-989.
37. Grosulescu, C., Juneja, V.K., Ravishankar, S., 2011. Effects and interactions of sodium lactate, sodium diacetate, and pediocin on the thermal inactivation of starved *Listeria monocytogenes* on bologna. Food Microbiology 28, 440-446.
38. Gutiérrez-Larraínzar, M., Rúa, J., Caro, I., de Castro, C., de Arriaga, D., García-Armesto, M.R., del Valle, P., 2012. Evaluation of antimicrobial and antioxidant activities of natural phenolic compounds against foodborne pathogens and spoilage bacteria. Food Control 26, 555-563.
39. Gutierrez, J., Barry-Ryan, C., Bourke, P., 2008. The antimicrobial efficacy of plant essential oil combinations and interactions with food ingredients. International Journal of Food Microbiology 124, 91-97.
40. Gutierrez, J., Barry-Ryan, C., Bourke, P., 2009. Antimicrobial activity of plant essential oils using food model media: Efficacy, synergistic potential and interactions with food components. Food Microbiology 26, 142-150.

41. Helander, I.M., Alakomi, H.L., Latva-Kala, K., Mattila-Sandholm, T., Pol, I., Smid, E.J., Gorris, L.G.M., Wright, A., 1998. Characterization of the action of selected essential oil components on gram-negative bacteria. Journal of agricultural and food chemistry 46, 3590-3595.
42. Hernández-Ochoa, L., Aguirre-Prieto, Y.B., Nevárez-Moorillón, G.V., Gutierrez-Mendez, N., Salas-Muñoz, E., 2011. Use of essential oils and extracts from spices in meat protection. Journal of Food Science and Technology, 1-7.
43. Honikel, K.-O., 2008. The use and control of nitrate and nitrite for the processing of meat products. Meat Science 78, 68-76.
44. Hunault, C.C., van Velzen, A.G., Sips, A.J., Schothorst, R.C., Meulenbelt, J., 2009. Bioavailability of sodium nitrite from an aqueous solution in healthy adults. Toxicology letters 190, 48-53.
45. Huq, T., Khan, A., Khan, R.A., Riedl, B., Lacroix, M., 2013. Encapsulation of probiotic bacteria in biopolymeric system. Food Science and Nutrition 53, 909-916.
46. Huq, T., Riedl, B., Bouchard, J., Salmieri, S. and Lacroix, M., 2014. Microencapsulation of Nisin in Alginate-Cellulose Nanocrystal (CNC) Microbeads for Prolonged Efficacy against Listeria monocytogenes. Cellulose.
47. Huq, T., Salmieri, S., Khan, A., Khan, R.A., Le Tien, C., Riedl, B., Fraschini, C., Bouchard, J., Uribe-Calderon, J., Kamal, M.R., Lacroix, M., 2012. Nanocrystalline cellulose (NCC) reinforced alginate based biodegradable nanocomposite film. Carbohydr Polym 90, 1757-1763.

48. Hyldgaard, M., Mygind, T., Meyer, R.L., 2012. Essential oils in food preservation: mode of action, synergies, and interactions with food matrix components. Frontiers in Microbiology 3.
49. Ibrahim Sallam, K., 2007. Antimicrobial and antioxidant effects of sodium acetate, sodium lactate, and sodium citrate in refrigerated sliced salmon. Food Control 18, 566-575.
50. Jayasena, D.D., Jo, C., 2013. Essential oils as potential antimicrobial agents in meat and meat products: A review. Trends in Food Science & Technology 34, 96-108.
51. Jebri, S., Hmaied, F., Jofre, J., MariemYahya, Mendez, J., Barkallah, I., Hamdi, M., 2013. Effect of gamma irradiation on bacteriophages used as viral indicators. Water Research 47, 3673-3678.
52. Jirovetz, L., Buchbauer, G., Stoilova, I., Stoyanova, A., Krastanov, A., Schmidt, E., 2006. Chemical Composition and Antioxidant Properties of Clove Leaf Essential Oil. Journal of agricultural and food chemistry 54, 6303-6307.
53. Jones, E., Salin, V., Williams, G.W., 2005. Nisin and the market for commercial bacteriocins. TAMRC Consumer and Product Research Report No. CP-01-05. http://afcerc. tamu. edu/publications/Publication-PDFs/CP% 2001% 2005% 20Nisin% 20 Report. pdf.
54. Jung, Y.J., Min, K.J., Yoon, K.S., 2009. Responses of acid-stressed *Salmonella Typhimurium* in broth and chicken patties to subsequent antimicrobial stress with ε-polylysine and combined potassium lactate and sodium diacetate. Food Microbiology 26, 467-474.

55. Kisko, G., Roller, S., 2005. Carvacrol and p-cymene inactivate Escherichia coli O157:H7 in apple juice. BMC Microbiol 5, 36.
56. Knowles, J.R., Roller, S., Murray, D.B., Naidu, A.S., 2005. Antimicrobial action of carvacrol at different stages of dual-species biofilm development by Staphylococcus aureus and Salmonella enterica serovar Typhimurium. Applied and Environtal Microbiology 71, 797-803.
57. Kotzekidou, P., 2013. Microbiological examination of ready-to-eat foods and ready-to-bake frozen pastries from university canteens. Food Microbiology 34, 337-343.
58. Lacroix, M., 2007. The use of essential oils and bacteriocins as natural antimicrobial and antioxidant compounds, Food, pp. 181-192.
59. Laird, K., Phillips, C., 2012. Vapour phase: a potential future use for essential oils as antimicrobials Letters in Applied Microbiology 54, 169-174.
60. Lopez, P., Sanchez, C., Batlle, R., Nerin, C., 2005. Solid-and vapor-phase antimicrobial activities of six essential oils: susceptibility of selected foodborne bacterial and fungal strains. Journal of agricultural and food chemistry 53, 6939-6946.
61. Manju, S., Jose, L., Srinivasa Gopal, T.K., Ravishankar, C.N., Lalitha, K.V., 2007. Effects of sodium acetate dip treatment and vacuum-packaging on chemical, microbiological, textural and sensory changes of Pearlspot (Etroplus suratensis) during chill storage. Food Chemistry 102, 27-35.
62. McGimpsey, J.A., Douglas, M.H., Van Klink, J.W., Beauregard, D.A., Perry, N.B., 1994. Seasonal variation in essential oil yield and composition from naturalized thymus vulgaris L. in New Zealand. Flavour and Fragrance Journal 9, 347-352.

63. Mead, P.S., Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M., Tauxe, R.V., 1999. Food-related illness and death in the United States. Emerging infectious diseases 5, 607.

64. Mello da Silveira, S., Luciano, F.B., Fronza, N., Cunha Jr, A., Scheuermann, G.N., Vieira, C.R., Chemical composition and antibacterial activity of Laurus nobilis essential oil towards foodborne pathogens and its application in fresh Tuscan sausage stored at 7 °C. LWT - Food Science and Technology.

65. Mello da Silveira, S., Luciano, F.B., Fronza, N., Cunha Jr, A., Scheuermann, G.N., Vieira, C.R., 2014. Chemical composition and antibacterial activity of *Laurus nobilis* essential oil towards foodborne pathogens and its application in fresh Tuscan sausage stored at 7° C. LWT-Food Science and Technology.

66. Millette, M., Le Tien, C., Smoragiewicz, W., Lacroix, M., 2007. Inhibition of Staphylococcus aureus on beef by nisin-containing modified alginate films and beads. Food Control 18, 878-884.

67. Myers, K., Montoya, D., Cannon, J., Dickson, J., Sebranek, J., 2013. The effect of high hydrostatic pressure, sodium nitrite and salt concentration on the growth of *Listeria monocytogenes* on RTE ham and turkey. Meat Science 93, 263-268.

68. Nazer, A.I., Kobilinsky, A., Tholozan, J.L., Dubois-Brissonnet, F., 2005. Combinations of food antimicrobials at low levels to inhibit the growth of *Salmonella* sv. Typhimurium: a synergistic effect Food Microbiology 22, 391-398.

69. Nedorostova, L., Kloucek, P., Kokoska, L., Stolcova, M., Pulkrabek, J., 2009. Antimicrobial properties of selected essential oils in vapour phase against foodborne bacteria. Food Control 20, 157-160.

70. Neetoo, H., Ye, M., Chen, H., 2010. Bioactive alginate coatings to control *Listeria monocytogenes* on cold-smoked salmon slices and fillets. International Journal of Food Microbiology 136, 326-331.
71. Nesbitt, A., Thomas, M.K., Marshall, B., Snedeker, K., Meleta, K., Watson, B., Bienefeld, M., 2014. Baseline for consumer food safety knowledge and behaviour in Canada. Food Control 38, 157-173.
72. Neves, P.R., McCulloch, J.A., Mamizuka, E.M., Lincopan, N., 2014. PSEUDOMONAS | Pseudomonas aeruginosa, in: Batt, C.A., Tortorello, M.L. (Eds.), Encyclopedia of Food Microbiology (Second Edition). Academic Press, Oxford, pp. 253-260.
73. Nyachuba, D., Donnelly, C., Howard, A., 2007. Impact of Nitrite on Detection of Listeria monocytogenes in Selected Ready to Eat (RTE) Meat and Seafood Products. Journal of Food Science 72, M267-M275.
74. Oussalah, M., Caillet, S., Lacroix, M., 2006. Mechanism of action of Spanish oregano, Chinese cinnamon, and savory essential oils against cell membranes and walls of *Escherichia coli* O157:H7 and *Listeria monocytogenes*. Journal of Food Protection 69, 1046-1055.
75. Oussalah, M., Caillet, S., Saucier, L., Lacroix, M., 2007. Inhibitory effects of selected plant essential oils on the growth of four pathogenic bacteria: *E. coli* O157:H7, *Salmonella* Typhimurium, *Staphylococcus aureus* and *Listeria monocytogenes*. Food Control 18, 414-420.
76. Perumalla, A., Hettiarachchy, N.S., Over, K.F., Ricke, S.C., Gbur, E.E., Zhang, J., Davis, B., 2012. Effect of Potassium Lactate and Sodium Diacetate Combination to Inhibit

Listeria Monocytogenes In Low and High Fat Chicken and Turkey Hotdog Model Systems. Open Food Science Journal 6, 16-23.

77. Phillips, C.A., Laird, K., Allen, S.C., 2012. The use of Citri-V™® — An antimicrobial citrus essential oil vapour for the control of Penicillium chrysogenum, Aspergillus niger and Alternaria alternata in vitro and on food. Food Research International 47, 310-314.
78. Porres-Martínez, M., González-Burgos, E., Accame, M.E.C., Gómez-Serranillos, M.P., 2013. Phytochemical composition, antioxidant and cytoprotective activities of essential oil of Salvia lavandulifolia Vahl. Food Research International 54, 523-531.
79. Quirós-Sauceda, A.E., Ayala-Zavala, J.F., Olivas, G.I., González-Aguilar, G.A., 2014. Edible coatings as encapsulating matrices for bioactive compounds: a review. Journal of Food Science and Technology, 1-12.
80. Ramaswamy, V., Cresence, V.M., Rejitha, J.S., Lekshmi, M.U., Dharsana, K.S., Prasad, S.P., Vijila, H.M., 2007. Listeria--review of epidemiology and pathogenesis. Journal of Microbiology, Immunology and Infection 40, 4-13.
81. Rasooli, I., Rezaei, M.B., Allameh, A., 2006. Ultrastructural studies on antimicrobial efficacy of thyme essential oils on *Listeria monocytogenes*. International Journal of Infectious Diseases 10, 236-241.
82. Redondo-Solano, M., Valenzuela-Martinez, C., Cassada, D.A., Snow, D.D., Juneja, V.K., Burson, D.E., Thippareddi, H., 2013. Effect of meat ingredients (sodium nitrite and erythorbate) and processing (vacuum storage and packaging atmosphere) on germination and outgrowth of Clostridium perfringens spores in ham during abusive cooling. Food Microbiology 35, 108-115.

83. Sales, E.H., Sedeh, F.M., Rajabifar, S., 2012. Effects of gamma irradiation and silver nano particles on microbiological characteristics of saffron, using hurdle technology. Indian journal of microbiology 52, 66-69.

84. Samaxa, R.G., Matsheka, M.I., Mpoloka, S.W., Gashe, B.A., 2012. Prevalence and Antimicrobial Susceptibility of *Salmonella* Isolated from a Variety of Raw Meat Sausages in Gaborone (Botswana) Retail Stores. Journal of Food Protection 75, 637-642.

85. Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R.V., Widdowson, M.-A., Roy, S.L., Jones, J.L., Griffin, P.M., 2011. Foodborne illness acquired in the United States major pathogens. Emerg Infectous Diseases 17.

86. Severino, R., Vu, K.D., Donsì, F., Salmieri, S., Ferrari, G., Lacroix, M., 2014. Antimicrobial effects of different combined non-thermal treatments against *Listeria monocytogenes* in broccoli florets. Journal of Food Engineering 124, 1-10.

87. Sindelar, J.J., Milkowski, A.L., 2011. Sodium nitrite in processed meat and poultry meats: A review of curing and examining the risk/benefit of its use. American Meat Science Association White Paper Series, 1-14.

88. Sivropoulou, A., Papanikolaou, E., Nikolaou, C., Kokkini, S., Lanaras, T., Arsenakis, M., 1996. Antimicrobial and Cytotoxic Activities of Origanum Essential Oils. Journal of agricultural and food chemistry 44, 1202-1205.

89. Solomakos, N., Govaris, A., Koidis, P., Botsoglou, N., 2008a. The antimicrobial effect of thyme essential oil, nisin and their combination against *Escherichia coli* O157:H7 in minced beef during refrigerated storage. Meat Science 80, 159-166.

90. Solomakos, N., Govaris, A., Koidis, P., Botsoglou, N., 2008b. The antimicrobial effect of thyme essential oil, nisin, and their combination against Listeria monocytogenes in minced beef during refrigerated storage. Food Microbiol 25, 120-127.
91. Solorzano-Santos, F., Miranda-Novales, M.G., 2012. Essential oils from aromatic herbs as antimicrobial agents. Current Opinion in Biotechnology 23, 136-141.
92. Sommer, R., Pribil, W., Appelt, S., Gehringer, P., Eschweiler, H., Leth, H., Cabaj, A., Haider, T., 2001. Inactivation of bacteriophages in water by means of non-ionizing (uv-253.7 nm) and ionizing (gamma) radiation: a comparative approach. Water Research 35, 3109-3116.
93. Stekelenburg, F.K., 2003. Enhanced inhibition of *Listeria monocytogenes* in Frankfurter sausage by the addition of potassium lactate and sodium diacetate mixtures. Food Microbiology 20, 133-137.
94. Tajkarimi, M.M., Ibrahim, S.A., Cliver, D.O., 2010. Antimicrobial herb and spice compounds in food. Food Control 21, 1199-1218.
95. Thomas, M.K., Murray, R., Flockhart, L., Pintar, K., Pollari, F., Fazil, A., Nesbitt, A., Marshall, B., 2013. Estimates of the burden of foodborne illness in Canada for 30 specified pathogens and unspecified agents, circa 2006. Foodborne Pathogens and Diseases 10, 639-648.
96. Turgis, M., Han, J., Caillet, S., Lacroix, M., 2009. Antimicrobial activity of mustard essential oil against *Escherichia coli* O157:H7 and Salmonella typhi. Food Control 20, 1073-1079.

97. Turgis, M., Vu, K.D., Dupont, C., Lacroix, M., 2012. Combined antimicrobial effect of essential oils and bacteriocins against foodborne pathogens and food spoilage bacteria. Food Research International 48, 696-702.

98. Tyagi, A.K., Malik, A., Gottardi, D., Guerzoni, M.E., 2012. Essential oil vapour and negative air ions: A novel tool for food preservation. Trends in Food Science & Technology 26, 99-113.

99. Weiss, J., Gaysinsky, S., Davidson, M., McClements, J., 2009. CHAPTER 24 - Nanostructured Encapsulation Systems: Food Antimicrobials, in: by, E., Colonna, G.B.-C.M.L.S.B. (Eds.), Global Issues in Food Science and Technology. Academic Press, San Diego, pp. 425-479.

100. Wijnker, J.J., Weerts, E.A.W.S., Breukink, E.J., Houben, J.H., Lipman, L.J.A., 2011. Reduction of Clostridium sporogenes spore outgrowth in natural sausage casings using nisin. Food Microbiology 28, 974-979.

101. Ye, H., Shen, S., Xu, J., Lin, S., Yuan, Y., Jones, G.S., 2013. Synergistic interactions of cinnamaldehyde in combination with carvacrol against food-borne bacteria. Food Control 34, 619-623.

102. Yi, S.M., Zhu, J.L., Fu, L.L., Li, J.R., 2010. Tea polyphenols inhibit Pseudomonas aeruginosa through damage to the cell membrane. International Journal of Food Microbiology 144, 111-117.

103. Zacharof, M., Lovitt, R., 2012. Bacteriocins produced by lactic acid bacteria a Review Article. APCBEE Procedia 2, 50-56.

104. Zhang, H., Kong, B., Xiong, Y.L., Sun, X., 2009. Antimicrobial activities of spice extracts against pathogenic and spoilage bacteria in modified atmosphere packaged fresh pork and vacuum packaged ham slices stored at 4°C. Meat Science 81, 686-692.

105. Zhou, G.H., Xu, X.L., Liu, Y., 2010. Preservation technologies for fresh meat – A review. Meat Science 86, 119-128.

106. http://www.about-ecoli.com

107. http://www.cdc.gov/hai/organisms/pseudomonas.html

108. http://www.inspection.gc.ca/food/information-for-consumers/fact-sheets/irradiation/eng/1332358607968/1332358680017

CHAPTER 6: APPENDICES

APP-1

Evaluating the efficiency of nitrite at two concentrations (100 and 200 ppm) against *Listeria monocytogenes in situ*

The maximum acceptable concentration for nitrite in food products is 200 ppm. The antimicrobial efficiency of nitrite was evaluated alone against *L. monocytogenes* in meat system (lean ground pork). The activity of maximum concentration of nitrite (200 ppm) was compared with the activity of half of that concentration (100 ppm). Table 1 demonstrates the antimicrobial activity of 100 ppm and 200 ppm of nitrite against *L. monocytogenes* during 7 days of storage.

Table 1: Final bacteria concentration (log CFU/g meat) in two different concentration of nitrite alone during 7 days of storage at 4 ^{o}C against *L. monocytogenes*

	Day 1	Day 4	Day 7
Control	3.23 ± 0.11 Ac[1]	3.96 ± 0.02 Bb	4.09 ± 0.25 Bb
100 ppm	2.91 ± 0.06 Ab	3.63 ± 0.06 Ba	3.74 ± 0.07 Ba
200 ppm	2.47 ± 0.14 Aa	3.56 ± 0.04 Ba	3.66 ± 0.03 Ba

[1]In the same column bearing the same lower case letters and in the same row bearing the same upper case letters is not significantly different ($p > 0.05$).

The results indicate that the antimicrobial activity of these two concentrations of nitrite was not significantly different after 4 and 7 days of storage. Indeed, results demonstrated that, using nitrite alone as an antimicrobial agent is not sufficient to get the high inhibition of bacteria.

APP-2

Sensorial analysis of organic acid salts in meat

The antimicrobial activity of mixture of organic acid salts (Potassium lactate 2.7% and Sodium acetate 0.4%) was evaluated and the results illustrated that the combination of organic acid salts has antimicrobial activity against total flora in ground pork (results are not shown). This study was conducted to find if the concentration of organic acid salts in ground meat is organoleptically acceptable in terms of smell and taste or not. Table 1 showed the concentration that we used in this study.

Table 1. Concentration of each component for mixture of organic acid salts

Control	Low concentration 1.55% (Potassium lactate 1.35% and Sodium acetate 0.2%)	High concentration 3.1% (Potassium lactate 2.7% and Sodium acetate 0.4%)

The ground meat was cooked at 400° F (~200 °C) for 10-15 minutes and 15 g of each sample was served warm to panelists. Each sample was coded with 3-digit random number. The samples were in small cups with the lid so the examiners shook the cup to release the smell and then they ate the meat. The panelists scored the sensory odor and taste of samples by using 9-point hedonic scale (9= Like extremely, 8=Like very much, 7=Like moderately, 6=Like slightly, 5=Neither like nor dislike, 4=Dislike slightly, 3=Dislike moderately, 2=Dislike very much, 1=Dislike extremely). They also had water and unsalted biscuit to drink and eat between the samples. The evaluation form was prepared and 10 examiners were asked to evaluate the smell and taste of samples. (The evaluation form is attached)

The results of smell and taste of organic acid salts in ground beef are presented in Table 2.

Table 2. The organoleptic properties of organic acid salts

	Smell	Taste
Control	6.75 ± 1.28	6.58 ± 1.74
Low concentration 1.55 %	7.75 ± 1.03	7.33 ± 2.25
High concentration 3.1	6.43 ± 2.06	5.33 ± 2.58

According to the hedonic scale, the scores above 5 are considered acceptable. So for organic acid salts both of the concentrations which were used were organoleptically accepted.

Âge :
Niveau scolaire :
Origine :
Heure de dégustation :

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ÉVALUATION SENSORIELLE

Merci de lire la présentation jusqu'à la fin avant de commencer l'évaluation. Si vous avez des questions, n'hésitez pas à les poser.

Lors de la dégustation d'aujourd'hui, il vous sera demandé d'évaluer les qualités organoleptiques de la viande.

Pour chacun de ces produits, trois échantillons vous seront présentés et deux critères seront étudiés (odeur et goût). Cette évaluation sera faite selon une échelle hédonique en 9 points et vous devrez encercler la réponse qui décrit le mieux l'impression que vous ressentez. Il n'y a pas de bonne ou de mauvaise réponse !

<u>Consignes spécifiques</u>

1/ Veuillez goûter les échantillons présentés en commençant par celui situé le plus à gauche pour finir par celui de droite.

2/ Entre chaque échantillon, veuillez manger un biscuit et prendre une gorgée d'eau.

3/ Pour évaluer l'odeur, veuillez secouer le contenant, ouvrir doucement le couvercle puis sentir l'échantillon.

Merci pour le temps que vous consacrez à cette étude !

Âge :
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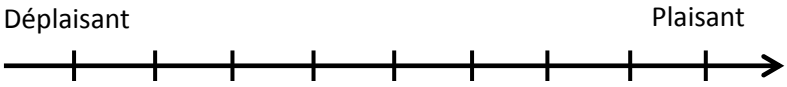
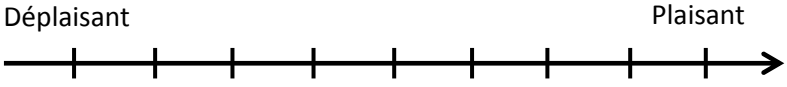
ANALYSE SENSORIELLE DE LA VIANDE

Échantillon 390



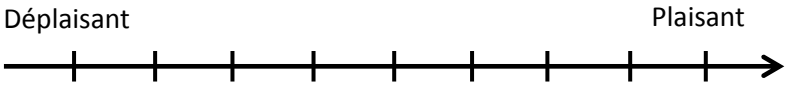
Odeur	1	2	3	4	5	6	7	8	9
Goût	1	2	3	4	5	6	7	8	9

Échantillon 754



Odeur	1	2	3	4	5	6	7	8	9
Goût	1	2	3	4	5	6	7	8	9

Échantillon 686



Odeur	1	2	3	4	5	6	7	8	9
Goût	1	2	3	4	5	6	7	8	9

Commentaires :_____
_____
_____
_____
_____